Evaluación de la participación de genes de respuesta al déficit hídrico en el aumento de tolerancia de las plantas micorrizadas frente a la sequía

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TESIS DOCTORAL

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DEPARTAMENTO DE MICROBIOLOGÍA DE SUELOS
Y SISTEMAS SIMBIÓTICOS.
ESTACIÓN EXPERIMENTAL DEL ZAIDÍN, C.S.I.C.
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El trabajo que se presenta en esta memoria de Tesis Doctoral se ha realizado en el Grupo de Micorrizas del Departamento de Microbiología de Suelos y Sistemas Simbióticos de la Estación Experimental del Zaidín (Granada), del Consejo Superior de Investigaciones Científicas con una beca asociada al proyecto "Bases moleculares de la tolerancia a estreses osmóticos en plantas micorrizadas" (AGL2002-03952).

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PORCEL, R; RUIZ-LOZANO, J.M. Arbuscular mycorrhizal influence on leaf water potential, solute accumulation and oxidative stress in soybean plants subjected to drought stress. ***Journal Experimental of Botany*** (2004) Vol. 55. 403:1743-1750

PORCEL, R; RUIZ-LOZANO, J.M. Evaluation of the role of genes encoding for Δ^1-pyrroline-5-carboxylate synthetase (P5CS) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants. ***Physiological and Molecular Plant Pathology*** (2004) 65: 211-221

PORCEL, R; RUIZ-LOZANO, J.M. 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. ***Journal of Experimental Botany*** (2004) Vol. 56. No 417: 1933-1942

PORCEL, R; GÓMEZ, M; KALDENHOFF, R; RUIZ LOZANO, J.M Impairment of NtAQP1 gene expression in tobacco plants does not affect root colonisation pattern by arbuscular mycorrhizal fungi but decreases their symbiotic efficiency under drought. ***Mycorrhiza*** (2005) 15: 417-423

PORCEL, R; AROCA, R; AZCÓN, R; RUIZ-LOZANO, J.M. PIP aquaporin gene expression in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants in relation to drought stress tolerance. ***Plant Molecular Biology*** (2006) (En prensa)

PORCEL, R; AROCA, R; CANO, C; BAGO, A; RUIZ-LOZANO, J.M. Identification of a gene from the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* encoding for a 14-3-3 protein, that is up-regulated by drought stress during the AM symbiosis. ***Mycrobial Ecology*** (2006) (En prensa).

PORCEL, R; AROCA, R.; CANO, C; BAGO, A; RUIZ-LOZANO, J.M. A gene from the arbuscular mycorrhizal fungus *Glomus intraradices* encoding a binding protein is up-regulated by drought stress during the mycorrhizal symbiosis. ***Environmental and Experimental Botany*** (Enviado)

Así mismo, parte de los resultados de esta Tesis Doctoral han sido presentados en los siguientes Congresos y Reuniones Científicas:

- **Cost 838 Meeting AM Research in Europe**. Pisa. Italia (Octubre, 2002)
- **VI Simposium Hispano-Portugués de Relaciones Hídricas en las Plantas**. Pamplona. España (Octubre, 2002)
- **Curso "Molecular Technology for Arbuscular Mycorrhizal Fungi"** under the auspices of de European Union RTD project, GENOMYCA (QLK5-CT-2000-01319/QLRT-2002-02804). The Scottish Agricultural College (SAC). Edimburgo. Escocia (Julio,2003)
- **II Curso Nacional de Genética**. Sociedad Española de Genética (SEG). Carmona, Sevilla. (Junio, 2004)
- **Cost 838 Meeting AM Research in Europe**. Granada. España (Octubre, 2004)
- **Cost Meeting**. Dijon, Francia (Junio, 2005)

La sequía es un fenómeno hidrológico extremo que puede definirse como una disminución coyuntural significativa de los recursos hídricos durante un período suficientemente prolongado que afecta a un área extensa con consecuencias socioeconómicas adversas. La sequía es un fenómeno "normal" y recurrente del clima, que ocurre en todas las regiones climáticas, pero sus características varían de unas regiones a otras. En cualquier caso, no se trata únicamente de un fenómeno físico o un evento de la naturaleza, sino que tiene efectos económicos y medioambientales importantes tanto en países en vía de desarrollo como en países desarrollados, que hacen patente la vulnerabilidad de las sociedades ante este fenómeno de la naturaleza.

En la Tierra, existen grandes zonas afectadas por la sequía, siendo muy común en regiones áridas y semi-áridas donde las lluvias son escasas y erráticas, con tasas de evapotranspiración que exceden a las precipitaciones. Además el cambio climático global que está afectando a nuestro planeta es el responsable de que numerosas áreas terrestres estén sometidas a periodos de sequía cada vez más frecuentes y de mayor duración, efecto que es especialmente palpable en las regiones de clima mediterráneo. En la actualidad España se encuentra inmersa en una situación de sequía. La última situación similar se prolongó entre los años 1990 y 1995, periodo en el que las lluvias entre los meses de Octubre y Mayo fueron, excepto en 1991-92, incluso superiores a las que se han registrado en el año 2005. Los meteorólogos consideran que la situación de sequía que afecta a España se suele «producir con una regularidad de diez años» y que el fenómeno es aleatorio, aunque guarda relación con el fenómeno global de cambio climático.

A pesar de llevar muchas décadas de investigación en agricultura, la sequía continua siendo un reto para las ciencias agrarias. La sequía afecta a muchos procesos nutricionales, fisiológicos y bioquímicos en las plantas y causa una reducción de las cosechas que oscila entre el 10 y el 90%, dependiendo de la severidad y el estadío de la planta en el momento de sufrir sus efectos. Desde el punto de vista genético, la resistencia a la sequía es un proceso complejo porque el funcionamiento de una especie vegetal o cultivar depende de la severidad, la duración y el estadío de desarrollo de la planta. Para complicar el tema, la sequía interacciona frecuentemente con otros estreses abióticos y bióticos.

Durante las últimas tres décadas la investigación sobre la simbiosis micorrícico arbuscular (MA) y sus repercusiones en agricultura ha incrementado notablemente su interés entre la comunidad científica, tanto desde la perspectiva de la nutrición de las plantas como de la tolerancia a sequía, a las bajas temperaturas, el biocontrol de patógenos de raíz o a sus implicaciones en fitoremediación. Además, el grado de respuesta de las plantas a la simbiosis MA es particularmente significativo en regiones áridas y semi-áridas donde la producción vegetal está normalmente limitada por deficiencias nutricionales e hídricas.

Diversos estudios a nivel ecofisiológico sobre el papel de la simbiosis MA frente al déficit hídrico han demostrado que dicha simbiosis origina una alteración en las tasas de movimiento de agua dentro, hacia y fuera de las plantas hospedadoras, con los consiguientes efectos sobre la hidratación de los tejidos y la fisiología de la planta. En los primeros trabajos sobre este tema, se concluía que la simbiosis MA probablemente afectaba las relaciones hídricas de las plantas de una forma indirecta, a través de una mejora de la nutrición fosforada de las mismas. Esta idea de que los efectos de las MA sobre las relaciones hídricas de las plantas eran de naturaleza meramente nutricional prevaleció durante varios años. Sin embargo, otros estudios posteriores demostraron que las relaciones hídricas e intercambio gaseoso de las plantas podía ser afectada por la simbiosis MA de forma independiente de la nutrición fosforada, y esto supuso un paso decisivo en los estudios de relaciones hídricas en la simbiosis MA. Así, hoy en día está totalmente aceptado que la simbiosis MA modifica las relaciones hídricas de las plantas, de una forma totalmente independiente del incremento en la captación de P. Los estudios realizados a nivel ecofisiológico en las últimas décadas han permitido sugerir posibles mecanismos a través de los cuales la simbiosis micorrícica incrementa la tolerancia de las plantas frente a la limitación hídrica. No obstante, estos mecanismos son muy complejos y las bases moleculares de los mecanismos relacionados con la tolerancia a la sequía de las plantas hospedadoras no se conocen aún. Lo que si está claro es que la simbiosis MA afecta a la fisiología de la planta hospedadora como resultado de una interacción molecular entre ambos miembros de la simbiosis y que se requiere un esfuerzo encaminado a orientar las investigaciones hacia la comprensión de la regulación molecular de dicha simbiosis. Es por ello que el objetivo principal de la presente Tesis Doctoral es el evaluar la posible implicación de un determinado grupo de genes caracterizados por ser genes de respuesta al déficit hídrico, en la mayor tolerancia de las plantas micorrizadas frente a la sequía. El trabajo se inicia con la determinación de los mecanismos fisiológicos y bioquímicos implicados en la protección de leguminosas frente a la sequía.

Objetivos específicos:

1) Determinar las bases de la protección de leguminosas frente al fenómeno de senescencia nodular prematura inducida por sequía.
2) Evaluar la posible participación de de un conjunto de genes, conocidos por su respuesta al déficit hídrico, en el aumento de la tolerancia de plantas micorrizadas frente al mismo. Este conjunto de genes incluye:
    a) Genes que codifican proteínas LEA
    b) Genes que codifican P5CS
    c) Genes que codifican AQP
    d) Gen fúngico que codifica una proteína 14-3-3
    e) Gen fúngico que codifica una proteína BiP

1. EL AGUA EN LA NATURALEZA

1.1. Introducción

El agua, al mismo tiempo que constituye el líquido más abundante en la Tierra, representa el recurso natural más importante y la base de toda forma de vida. De este modo, desde el punto de vista de la agricultura, la falta de agua es el factor limitante de la producción vegetal de mayor importancia a escala global terrestre (Kramer y Boyer, 1997).

No es usual encontrar el agua en forma pura, aunque en el laboratorio puede llegar a obtenerse o separarse en sus elementos constituyentes, que son el hidrógeno (H) y el oxígeno (O). Cada molécula de agua está formada por un átomo de oxígeno y dos de hidrógeno, unidos fuertemente en la forma H-O-H. A diferencia de los hidruros no metales, que son gases a temperatura ambiente, el agua es líquida. La razón de ello y de la mayoría de las propiedades poco corrientes del agua es que sus moléculas son polares y forman puentes de hidrógeno entre sí, lo que ocasiona un aumento de las temperaturas de fusión y ebullición. La cohesión, otra propiedad del agua, es la responsable de la unión entre las moléculas de agua y por tanto, de que las columnas finas de agua puedan ascender por los vasos xilemáticos sin romperse hasta la cima de un árbol. También es la responsable de que se requiera una cantidad de energía muy elevada para provocar la evaporación (Azcón-Bieto y Talón, 1993)

El agua permanece en constante movimiento. Desde los mares, ríos, lagos, e incluso desde los seres vivos, se evapora agua constantemente hacia la atmósfera, hasta que llega un momento en que ese agua se precipita de nuevo hacia el suelo. De este agua que cae, una parte se evapora, otra se escurre por la superficie del terreno hasta los ríos, lagos, lagunas y océanos, y el resto se infiltra en las capas de la tierra, y fluye también subterráneamente hacia ríos, lagos y océanos. Este agua subterránea es la que utilizan los vegetales, los cuales la devuelven después de nuevo a la atmósfera. Como observamos, al volver el agua a la atmósfera se completa un ciclo, que se denomina ciclo hidrológico (Figura 1). De esta manera la naturaleza garantiza que el agua no se pierda y pueda volver siempre a ser utilizada por los seres vivos.

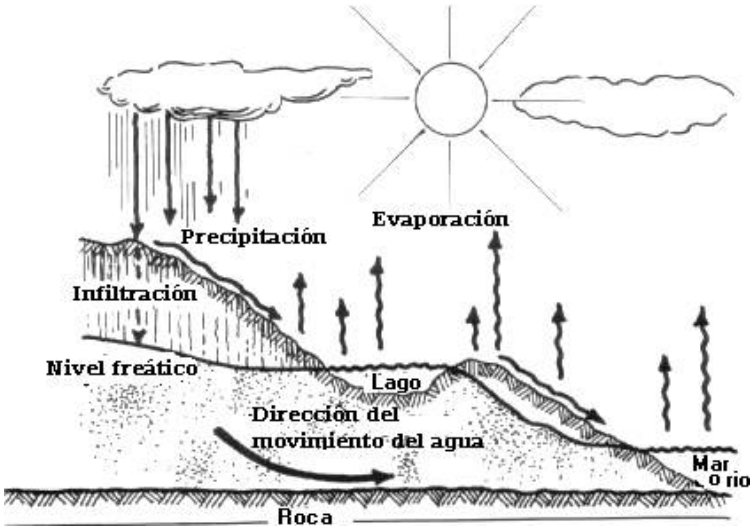


Figura 1. Esquema del ciclo hidrológico

La vida en la Tierra ha dependido siempre del agua. Sirve de hábitat a una gran parte de los organismos y además, desempeña un importante papel en la fotosíntesis de las plantas. El agua es un disolvente para muchas sustancias tales como sales orgánicas, azúcares y aniones orgánicos y constituye un medio en el cual tienen lugar todas las reacciones bioquímicas. En su forma líquida, permite la difusión y el flujo masivo de solutos, y por esta razón, es esencial para el transporte y distribución de nutrientes y metabolitos en toda la planta. También es importante el agua en las vacuolas de las células vegetales, ya que ejerce presión sobre el protoplasto y la pared celular, manteniendo así la turgencia en hojas, raíces y otros órganos de la planta. Es el componente mayoritario en la planta (aproximadamente un 80-90% del peso fresco en plantas herbáceas y más de un 50% de las partes leñosas) y afecta, directa o indirectamente, a la mayoría de procesos fisiológicos.

1.2. Relaciones hídricas en plantas

1.2.1. Cuantificación del estado hídrico de la planta

Como base para comprender las relaciones planta-agua, se hace necesario definir y determinar el estado hídrico a nivel celular, de órgano o incluso, de planta entera.

El estado hídrico de las plantas se puede estudiar en términos de **contenido hídrico** (CH), expresado como porcentaje del peso seco:

$$CH = \frac{P_f - P_s}{P_s} \times 100$$

donde:
P_f, peso fresco de la muestra
P_s, peso seco de la muestra, determinado después de mantenerla en estufa a 80 °C durante 24 horas.

No obstante, debido a que el peso seco puede experimentar cambios diarios y estacionales, las determinaciones comparativas del contenido hídrico basadas en el peso seco no son satisfactorias. Una forma de eliminar estos problemas consiste en expresar el contenido hídrico sobre la base del contenido hídrico a plena turgencia, es decir, al peso turgente (P_t), pasando a denominarse **contenido hídrico relativo** (CHR) (Boyer, 1995)

$$CHR = \frac{P_f - P_s}{P_t - P_s} \times 100$$

El P_t se obtiene tras incubar los tejidos de la planta en agua destilada y temperatura ambiente durante 2-3 horas.

La magnitud que se utiliza para expresar el contenido de energía libre del agua es el potencial hídrico (Ψ). El potencial hídrico (Ψ) se define como la energía libre por unidad de volumen de agua (Boyer, 1995) y se asume que el potencial hídrico del agua es 0 en condiciones estándar (temperatura ambiente y presión atmosférica).

Dado que la energía/unidad de volumen tiene las mismas dimensiones que la presión, el Ψ de la planta y el suelo se expresan en unidades de presión, normalmente MPa. El Ψ disminuye por debajo del Ψ del agua pura por la existencia de solutos disueltos, por la existencia de unas fuerzas de presión ejercidas por el ambiente sobre el agua, por la fuerza de la gravedad y también por la unión de las moléculas de agua a superficies debido a fuerzas matriciales. Por ello el Ψ del agua en el sistema suelo-planta-atmósfera se expresa como:

$$\Psi = \Psi_s + \Psi_p + \Psi_g + \Psi_m$$

donde:
Ψ_s = potencial de solutos
Ψ_p = potencial de presión
Ψ_g = potencial gravitacional
Ψ_m = potencial mátrico

No obstante, el potencial gravitacional puede tener un efecto sustancial sobre el potencial hídrico cuando el agua es transportada a distancias verticales superiores a 5-10 m, pero este componente puede ser omitido cuando se trata de transporte de agua entre células o en plantas pequeñas. Por otro lado, el potencial mátrico, representa el efecto de las superficies de paredes y componentes celulares en la retención de agua. Tiene valores muy bajos y difíciles de medir, por lo que es complicado determinar el impacto de este componente sobre el potencial hídrico del agua en el suelo o en la planta y se suele ignorar. No obstante, en células con elevada proporción de matriz no se puede ignorar pues su contribución al potencial hídrico es significativa. Por todo ello, la ecuación más frecuente para expresar el potencial hídrico es (Boyer, 1995):

$\Psi = \Psi_s + \Psi_p$

donde:
Ψ_s está determinado por la concentración de sustancias osmóticamente activas en el agua. En una célula vegetal este componente tiene siempre valores negativos.
Ψ_p representa la presión que la pared celular ejerce sobre el interior celular y en una célula vegetal siempre muestra valores positivos.

1.2.2. El movimiento de agua en el sistema suelo-planta-atmósfera

El abastecimiento de agua es un factor esencial en la vida de la planta. La pérdida de agua por las hojas en la transpiración es un proceso inevitable pero necesario para la vida vegetal, ya que, entre otros, contribuye a constituir un sistema circulatorio abierto y posibilita el intercambio de CO_2 para la fotosíntesis. Para compensar esta pérdida, las raíces absorben agua a partir del suelo con lo cual se establece un flujo de agua a través de la planta, desde el suelo hasta la atmósfera (Estaún, 1991)

El suelo es un sistema complejo que consta de la matriz sólida así como la disolución del suelo y del aire que ocupa el espacio poroso. El tamaño y naturaleza química de las partículas determinan cuánta agua se puede retener en el suelo en contra de la acción de la gravedad y con qué fuerza se retiene. Esta capacidad de retener el agua depende del potencial mátrico (Boyer, 1995).

Cuando un suelo está totalmente mojado después de haber drenado por gravedad, estado descrito como capacidad de campo, su potencial hídrico es cero. No obstante, a medida que el suelo se seca, bien por evaporación en su superficie, o bien porque las raíces absorben agua, desciende el potencial mátrico y el potencial hídrico se hace más negativo. Se denomina porcentaje de marchitez permanente el contenido hídrico del suelo con el cual las plantas se marchitan. Aunque existen algunas excepciones, la mayoría de las plantas son incapaces de extraer cantidades importantes de agua cuando el potencial hídrico del suelo se hace inferior a –1,5 MPa. Tradicionalmente se ha definido como agua disponible la que existe entre la capacidad de campo y el porcentaje de marchitez permanente. Hay que decir, no obstante, que en este margen el agua no está uniformemente disponible. En un suelo que se seca, las plantas empezarán a mostrar signos de

déficit hídrico y reducción del crecimiento mucho antes de que el potencial hídrico del suelo alcance el porcentaje de marchitez permanente.

Un aspecto general sobre el movimiento del agua en las plantas es que es un proceso totalmente "pasivo". En general, existen dos tipos de movimiento del agua: flujo masivo y difusión. El flujo masivo es el movimiento de moléculas de agua y solutos de manera conjunta y en una dirección, debido a diferencias de presión. Por el contrario, el movimiento de agua entre o hacia las células vivas, o a través del suelo, tiene lugar mediante difusión.

Una contribución importante al estudio de las relaciones hídricas en las plantas es el tratamiento del movimiento de agua desde el suelo hacia las raíces, a través de la planta y hacia el aire, como una serie de procesos estrechamente interrelacionados (Sánchez-Díaz y Aguirreolea, 2002b). El movimiento de agua en el continuo suelo-planta-atmósfera (CSPA) se puede describir como:

$$\textbf{Flujo} = \frac{\text{diferencia de } \Psi}{\text{resistencia}}$$

Este concepto se puede aplicar al flujo en equilibrio estable a través de la planta de la manera siguiente:

$$\textbf{Flujo} = \frac{\Psi_{suelo} - \Psi_{raíz}}{r_1} = \frac{\Psi_{raíz} - \Psi_{tallo}}{r_2} = \frac{\Psi_{tallo} - \Psi_{hoia}}{r_3} = \frac{C_{hola} - C_{aire}}{r_{hoja} + r_{aire}}$$

donde:
r_1, r_2 y r_3 constituyen las resistencias en las partes respectivas de la vía y *C* corresponde a la concentración de vapor de agua.

El concepto de continuo proporciona una teoría útil y unificadora en la cual el movimiento del agua a través del suelo, raíces, tallos y hojas, así como su evaporación hacia el aire, se pueden estudiar en términos de las fuerzas motrices y las resistencias que actúan en cada segmento. El concepto también es útil para analizar la manera en que diversos factores del ambiente afectan al movimiento del agua. Así por ejemplo, la sequía en el suelo provoca tanto un aumento en la resistencia al flujo de agua hacia las raíces como una disminución del potencial hídrico; una aireación deficiente y una disminución de la temperatura del suelo aumentan la resistencia al flujo de agua en las raíces, y un aumento en la temperatura de la hoja y del aire incrementa la transpiración al aumentar el gradiente de concentración de vapor de agua o fuerza motriz desde la hoja al aire. Asimismo, el cierre de los estomas aumenta la resistencia a la difusión del vapor de agua fuera de las hojas (Nilsen y Orcutt, 1996).

En cualquier caso, el movimiento del agua en la planta se presenta a lo largo de gradientes de disminución de energía libre, expresado normalmente como

diferencias de Ψ. El agua se mueve desde zonas de mayor a otras de menor potencial hídrico. En el interior de la planta, Ψ es más elevado en las raíces, disminuyendo progresivamente en el tallo, observándose los valores más bajos en las hojas (Figura 2)

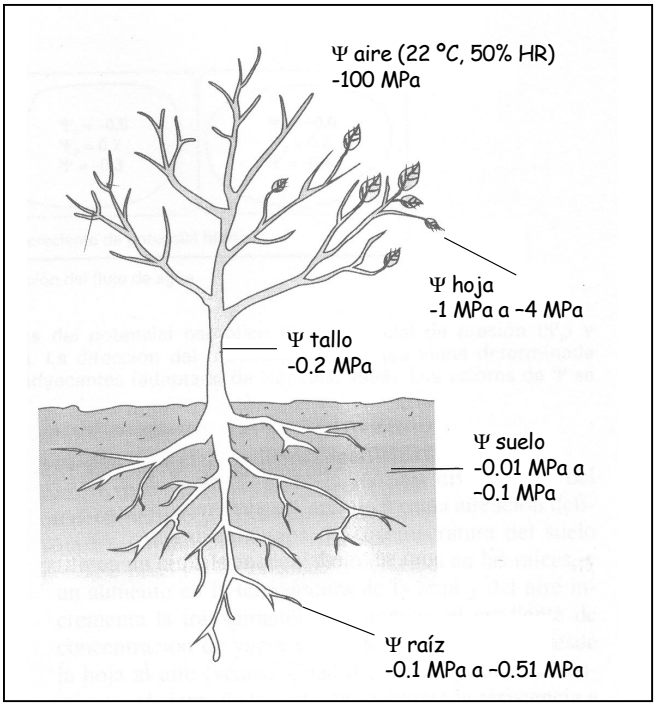


Figura 2. Potencial hídrico en el sistema suelo-planta-atmósfera. El valor más negativo de Ψ se encuentra en la atmósfera. En la planta existe un gradiente, siendo el potencial más negativo en las hojas y menos negativo en las raíces, donde Ψ se aproxima al del suelo. (Tomado de Azcón-Bieto y Talón, 2002)

El agua entra en las raíces en respuesta a un gradiente de potencial hídrico en el xilema, establecido por la transpiración. Claramente, el agua entrará con mayor rapidez a través de aquellas regiones de la raíz que ofrezcan menor resistencia. El agua que entra en una raíz primaria ha de atravesar, primero, la epidermis e hipodermis y una capa cortical parenquimática. Seguidamente, pasa a la endodermis con la banda de Caspari y, una vez en su interior, se mueve a través de una capa de periciclo, antes de alcanzar el tejido vascular.

Teóricamente, existen tres vías anatómicamente diferentes a través de las cuales podría moverse el agua. En primer lugar, la ruta externa al citoplasma, que se denomina **apoplasto**. En segundo lugar, existe la posibilidad de que el agua atraviese la pared celular y el plasmalema, para entrar luego en el citoplasma. Esta ruta, que representa la porción viva de la célula, se denomina vía del **simplasto**. Finalmente, existe la posibilidad de que el agua atraviese tanto el plasmalema como el tonoplasto, de tal manera que la vacuola pasaría a ser una parte integral de la vía de transporte (Sánchez-Díaz y Aguirreolea, 2002b)

El **xilema** es el tejido a través del cual el agua asciende en el tallo. En angiospermas, el agua se mueve primordialmente a través de **vasos**, mientras que en gimnospermas, el elemento conductor es la **traqueida**. Las células de vasos y traqueidas pierden el citoplasma durante su maduración y poseen paredes celulares muy lignificadas y relativamente rígidas. Existen conexiones frecuentes entre las hileras adyacentes de células a través de punteaduras en las paredes laterales. Los vasos y las traqueidas se extienden hacia las raíces y hojas, donde se ramifican repetidamente.

1.2.3. Ascensión de agua en la planta

La ascensión del agua en la planta a través de los vasos xilemáticos se puede explicar mediante la teoría de la tensión-cohesión.

Cuando el agua se halla confinada en tubos con diámetro estrecho y paredes humedecibles (tales como los vasos y traqueidas xilemáticas), al aplicar una presión negativa desde la parte superior, la tensión se transmitirá a través de la columna de agua sin que se pierda el contacto con la pared del tubo (fuerzas de cohesión), es decir, las columnas de agua se comportan como si todas las moléculas estuviesen conectadas, y una tensión aplicada a cualquier parte de la columna se transmite a través de la misma, moviendo el agua hacia la fuente de la tensión.

La transpiración crea un gradiente de potencial hídrico a través del mesófilo foliar, que provoca que el agua desaparezca en los extremos de los nervios foliares. La pérdida de agua a ese nivel crea una tensión en las columnas del xilema, cuya magnitud depende de la intensidad transpiratoria y que es transmitida hasta la raíz, asegurándose así el movimiento del agua a través de toda la planta (Sánchez-Díaz y Aguirreola, 2002b)

1.3. La problemática: Sequía en el Mediterráneo

1.3.1. Introducción

La cuenca del Mediterráneo agrupa una serie de países más allá de los del Sur de Europa; también se incluyen los países del Norte de África y los de la costa Este del mar como Turquía, Líbano, Siria, Israel o Jordania (Fotografía 1). Aunque todos estos países son muy diferentes en los aspectos culturales y políticos, comparten un clima parecido con unas características muy particulares, que les hace coincidir en sus preocupaciones por el cambio climático y su impacto sobre la agricultura, que es todavía un sector económico muy importante en toda el área. Una solución común para todos ellos sería la mejora del uso del agua en este sector.

Fotografía 1. En esta imagen de satélite se puede ver la cuenca del Mediterráneo y cómo el color verde, muy abundante en el Norte de Europa (son las áreas con más vegetación), se vuelven poco a poco más marrones a medida que bajamos hacia el Sur y sobre todo en el Norte de África. Esto significa que la vegetación es más densa en las zonas más verdes y nos da una idea de cómo el agua está distribuida en el espacio. Índice de diferencia de vegetación normalizada (NDVI) G. Begni, MEDIAS-France

1.3.2. Aridez y sequía

Una **región árida** es aquella en la que las precipitaciones son escasas o nulas y la humedad atmosférica es muy baja. Sin embargo, una región puede tener un volumen total de precipitación anual que en otra sería suficiente para sostener un tapiz vegetal continuo y, no obstante ser árida; en el segundo caso, el agua precipitada, aunque escasa, es aprovechada íntegramente, al menos durante el período vegetativo. Intervienen factores ajenos a la precipitación que limitan considerablemente el aprovechamiento del agua. Entre estos factores destaca la temperatura, que condiciona directamente la intensidad de la evaporación. Por tanto, la noción de aridez hay que hacerla combinando los datos de precipitación con los de temperatura (Sánchez-Díaz y Aguirreolea, 2002a).

Desde el punto de vista tanto meteorológico como ambiental, podemos definir la **sequía** como la falta o insuficiencia de precipitación durante un período largo, que provoca un desequilibrio hidrológico considerable y, por tanto, restricción en el suministro de agua. Se presenta cuando la evaporación y la transpiración exceden la precipitación durante un tiempo considerable (Fotografía 2).

Fotografía 2. Vista aérea del desierto de Tabernas en Almería (izquierda). Imagen de un cultivo afectado por sequía (derecha)

1.3.3. La sequía en España: pasado y presente

España padece con cierta regularidad e intensidad, según las regiones, periodos de sequía. El carácter más o menos habitual de este fenómeno se debe a que la Península Ibérica pertenece al dominio climático mediterráneo y a que se encuentra próxima al ámbito de subsidencia subtropical del anticiclón de las Azores. Además, factores de naturaleza geográfica e hidrográfica explican la mayor frecuencia de este fenómeno en los archipiélagos de Baleares y Canarias, así como en el centro, sur y sureste peninsular. En cualquier caso, la sequía en nuestra península no es un fenómeno reciente como algunos podrían creer. Diversas crónicas medievales que han llegado hasta nosotros nos hablan de periodos de baja pluviometría. Durante el califato de Abderramán III, el territorio de Al-Andalus sufrió los rigores de la escasez de agua tal y como nos lo relata Ibn Hayyán en el *Muqtabas V* . Así mismo, España sufrió una terrible sequía entre 1567 y 1568. Aunque hubo una cierta recuperación a finales del siglo XVI, los rendimientos de las cosechas a principios del siglo XVII fueron empeorando y así podemos constatar dos grandes crisis agrarias: 1604-1605 y 1615-1617. Los inviernos de 1603-04 y 1604-05 fueron bastante secos en gran parte de la geografía peninsular (Barriendos, 1997).

La España del siglo XX también sufrió con mayor insistencia si cabe los rigores de este fenómeno. Una terrible sequía se produjo en 1930, lo cual acrecentó la tensión social y política que se venía arrastrando desde hacía varios años. Dos episodios secos relativamente próximos en el tiempo y situados en los años 1980-83 y 1990-94, pueden ilustrar algunos aspectos. La primera de ellas afectó a gran parte de la península ibérica, advirtiendo sus consecuencias directas en la actividad agraria -descenso de cosechas-, el aumento desorbitado de las perforaciones acuíferas, con claros síntomas de sobreexplotación y salinización de las aguas en las cuencas del centro y sur de España y con caracteres muy intensos en las del Júcar, Segura, Guadiana y Sur (Font, 1988).

El inicio de la década de los noventa coincide con un periodo de sequía, que sin duda sensibiliza a la opinión pública, creándose un debate desde el propio gobierno al enviar al Consejo Nacional del Agua, en abril de 1993, el anteproyecto de Plan Hidrológico Nacional.

Según los últimos datos ofrecidos por el Instituto Nacional de Meteorología el año hidrometeorólogico 2004-2005 se ha caracterizado por un acusado déficit de precipitaciones, incluso el mes de enero de 2005 ha sido uno de los meses más secos de los últimos 50 años, junto con los de los años 1983 y 1993.

La sequía en España es, por tanto, una situación característica de un país con un clima como el nuestro y lo que hay que hacer entre todos es aprender a convivir con la sequía, anticipándonos a sus consecuencias previsibles y gestionando la misma.

1.3.4. Déficit hídrico y crecimiento vegetal

El déficit hídrico es el factor más importante que causa la reducción en el crecimiento de la planta. Los déficit hídricos tienen efectos químicos e hidráulicos que afectan a gran variedad de procesos fisiológicos y bioquímicos en las plantas (Sánchez-Díaz y Aguirreolea, 2002a). Así, la perdida de agua en un tejido vegetal tiene los siguientes efectos que pueden influir en el metabolismo de la planta:

- Reducción del potencial hídrico o la actividad de agua celular
- Disminución de la presión turgente
- Concentración de moléculas, a medida que disminuye el volumen celular con la reducción de la turgencia
- Alteración de relaciones espaciales en el plasmalema, tonoplasto y membranas de orgánulos, debido a cambios de volumen
- Cambio de la estructura o configuración de las macromoléculas como consecuencia de la eliminación del agua de hidratación o modificación de la estructura de esa agua.

En cualquier caso, el efecto más importante de, incluso, un estrés hídrico suave es la reducción del crecimiento, siendo especialmente sensible la expansión celular. La división celular, aunque resulta afectada por el estrés hídrico, normalmente es menos sensible que la expansión celular. Además de una inhibición del crecimiento, los déficit hídricos modifican el desarrollo y la morfología vegetal como cambios en la relación raíz/parte aérea. Otro efecto sobre el desarrollo vegetativo es la terminación precoz del crecimiento en extensión de especies perennes, con la formación de yemas en reposo. Los déficit hídricos también provocan la abscisión de hojas y frutos, especialmente tras la desaparición del estrés. Además, afectan al desarrollo reproductor, necesitándose, en algunas especies, un período de sequía para estimular la iniciación floral o provocar la emergencia de yemas florales ya diferenciadas.

La disminución en la tasa respiratoria se acompaña siempre de una disminución en la absorción de CO_2 y, por tanto, de la fotosíntesis. Los déficit hídricos también afectan a la fotosíntesis a través de sus consecuencias en los procesos enzimáticos, transporte electrónico y contenido en clorofila. La

transpiración también se reduce como consecuencia de la inhibición del crecimiento de la parte aérea. Por el contrario, suele aumentar la capacidad absorbente de las raíces por unidad de superficie. Otros cambios que tienen lugar son: incremento de las reacciones degradativas en relación con las sintéticas, disminución de la síntesis de proteínas, aumento de la concentración de aminoácidos libres, especialmente prolina, glicina betaína, di y poliaminas y azúcares. Los tejidos sometidos a estrés hídrico presentan una disminución en los grupos –SH, y un aumento en la actividad peroxidasa y en la formación de peróxido de hidrógeno, lo que sugiere que los tejidos sometidos a estrés hídrico poseen un mayor estado de oxidación (Kramer y Boyer, 1997).

Por otra parte, la sequía puede inducir la transcripción de ARN mensajeros que codifican un gran número de proteínas y que no son sintetizadas en condiciones óptimas. Muchas de estas proteínas son también inducidas por el ácido abscísico (Sánchez-Díaz y Aguirreolea, 2002a).

2. SISTEMAS IMPLICADOS EN LA TOLERANCIA A LA SEQUÍA EN PLANTAS

2.1. Introducción

El déficit hídrico conlleva una serie de cambios morfológicos, fisiológicos, bioquímicos y moleculares que afectan negativamente al desarrollo y productividad de las plantas (Wang *et al.*, 2001). Aunque las distintas especies de plantas varían en su sensibilidad y respuesta a la disminución del potencial hídrico celular, se sabe que todas las plantas han adquirido la capacidad de percibir, señalizar y responder a la falta de agua (Bohnert *et al.*, 1995). Muchas de las condiciones ambientales adversas ejercen su efecto negativo, al menos en parte, generando a su vez un estrés oxidativo, que puede causar la desnaturalización de proteínas estructurales y funcionales (Smirnoff, 1998). A continuación se resumen los sistemas con los que cuenta la planta para reaccionar y defenderse frente al déficit hídrico en sus tejidos.

La complejidad de la adaptación al estrés: principales dianas en la ingeniería de tolerancia al estrés

Clase de diana	Ejemplos	Posible modelo(s) de acción
Osmoprotectores	Aminoácidos (prolina, ectoína) Compuestos dimetil-sulfonio (glicina-betaína, DMSP) Polioles (manitol, D-ononitol, sorbitol) Azúcares (sacarosa, trealosa, fructano)	Ajuste osmótico; protección proteína/membrana; secuestro reactivos (OH^-)
Secuestradores oxígeno reactivo	Enzimáticos (catalasa, Fe/Mn SOD, ascorbato peroxidasa, enzimas ciclo glutation; glutation-S-transferasa; glutation peroxidasa; no enzimáticos (ascorbato, flavonas, carotenoides...)	Detoxificación de especies reactivas del oxígeno
Proteínas del estrés	Proteínas LEA	Desconocido, estabilización proteinas, chaperonas, secuestro de iones, estabilización de proteína/membrana
Proteínas choque térmico	Varias proteínas de choque de frío, calor, salino en muchos compartimentos celulares	Modulación translacional
Transportadores ion/protón	Transportadores de alta afinidad por K^+; canales de baja afinidad por K^+; ATPasas de membrana plasmática, vacuolar pre-vacuolar y organular, y transportadores de iones (H^+-ATPasa, antiporter Na^+/K^+	Captación y transporte de K^+/Na^+; establecimiento de gradiente de protones; eliminación y secuestro de iones (tóxicos) del citoplasma y orgánulos
Fluidez de membrana	Acidos grasos desaturados	Cantidades de dienoico y fluidez incrementada; tolerancia al frío
Estatus hídrico	Aquaporinas o canales de agua (facilitadores de solutos: urea, glicerol, CO_2, y otros incluyendo iones); concentración CO_2	Regulación diferencial de la cantidad de AQP en tonoplasto y plasmalema; mejora de estomas
Componentes señalización	Homólogos de histidin-kinasas (AtRR1/2), MAP kinasas (HOG); Protein-kinasas dep de Ca^{2+}, inositol kinasas...	Señales de transducción mediadas por sensores Ca^{2+}/fosforilación
Control de transcripción	Factores de transcripción: EREBP/AP2 (DREB, CBF); dedo de zinc TF (Alfin 1); Myb (AtMyb2, CpMyb10)	Inducción/activación de la transcripción
Reguladores del crecimiento	Rutas biosintéticas alteradas o niveles conjugados de ácido abscísico, citokininas y/o brasinosteroides	Cambios en la homeostasis hormonal

Tabla 1. Principales dianas y posibles modelos de acción de la tolerancia al estrés en plantas. AP2, APETELA 2; AQP, aquaporina; AtMyb, factor de transcripción de mieloblastosis de *Arabidopsis thaliana*; AtRR1, reguladores de la respuesta a dos componentes de *Arabidopsis thaliana*; CBF, factor de unión-C/DRE; CpMyb, factor de transcripción de mieloblastosis de *C. plantagineum*; DMSP, dimetilsulfoniopropionato; DREB, proteína de unión (DRE) responsable de la deshidratación; TF, factor de transcripción. (Cushman y Bohnert, 2000).

Las plantas responden al estrés hídrico a nivel morfológico, anatómico y celular con modificaciones que conducen, bien a evitar el estrés, o a incrementar la tolerancia al mismo. Las adaptaciones morfológicas y anatómicas pueden ser vitales en determinadas especies vegetales, pero no son utilizadas de forma generalizada por todas las plantas. Por el contrario, las respuestas celulares al estrés hídrico si parecen conservadas en todas las plantas. El déficit hídrico celular puede originar una concentración de solutos, cambios en el volumen celular y en la forma de la membrana plasmática, perdidas del gradiente de potencial hídrico, del turgor o de la integridad de la membrana, y desnaturalización de proteínas, (Bohnert *et al.*, 1995). Por tanto, la capacidad de la totalidad de la planta para responder o sobrevivir al déficit hídrico celular, depende de mecanismos globales en sus tejidos que integren la respuesta celular al estrés (Bray, 1997).

2.2. Ajuste osmótico y solutos compatibles

Cuando disminuye el potencial osmótico de los tejidos en respuesta al desarrollo de un déficit hídrico interno, es importante distinguir el componente resultante de una concentración pasiva de solutos, debido a deshidratación del tejido, del originado por acumulación activa de los mismos. Es a este último caso de osmorregulación, al que se le denomina ajuste osmótico (Antolín y Sánchez-Díaz, 1992).

El ajuste osmótico posibilita así el mantenimiento, en condiciones de sequía, de la turgencia y de los procesos dependientes de la misma, tales como expansión y crecimiento celular, apertura estomática, fotosíntesis, etc. Por otra parte, retrasa el enrollamiento foliar y mantiene el crecimiento de la raíz. Ordinariamente, el ajuste osmótico se presenta en plantas sometidas a un estrés lento. Los solutos que participan varían pero, por lo general, además de iones inorgánicos (especialmente K^+ y Cl^-) y solutos orgánicos cargados eléctricamente que se acumulan en la vacuola, se sintetizan y acumulan en el citoplasma moléculas orgánicas sin carga específica, que reciben el nombre de solutos compatibles, siendo los dos más frecuentes, la prolina y la glicina-betaína. Se ha observado que, en condiciones de sequía, también se acumulan otras sustancias, tales como azúcares reductores, sacarosa o pinitol (Antolín y Sánchez-Díaz, 1992).

Algunos de los genes que se inducen por déficit hídrico codifican proteínas y enzimas implicadas en la síntesis de solutos compatibles. Los solutos compatibles se caracterizan por su bajo peso molecular, por ser eléctricamente neutros, muy solubles y, aunque se encuentren en altas concentraciones, no resultan tóxicos (Yancey, 1984). Éstos se acumulan en distintos organismos como respuesta al estrés osmótico actuando como osmoprotectores, lo que supone un mecanismo de adaptación al estrés hídrico y salino (Berstein, 1961; Hasegawa *et al.*, 2000). En plantas, estos solutos compatibles se acumulan principalmente en el citosol, en cloroplastos y otros orgánulos que en conjunto ocupan menos del 20% del volumen total de la célula (Rontein *et al.*, 2002). En plantas que acumulan osmolitos de forma natural, estos pueden alcanzar concentraciones de 6 a 60 mM. La concentración de osmolitos aumenta durante la exposición al estrés (Rhodes y

Hanson, 1993; Bohner *et al.*, 1995), llegando a alcanzar 200 mM, concentración osmóticamente significativa.

Los solutos compatibles se pueden clasificar en tres grupos: aminoácidos (prolina), aminas cuaternarias (glicina betaína) y poli alcoholes (manitol, pinitol, ononitol). No todos se producen en plantas. Un aspecto importante de estos compuestos es que sus efectos beneficiosos son generales independientemente de la especie vegetal.

2.2.1. Prolina

La acumulación de prolina en plantas es una respuesta al estrés osmótico bien conocida (Singh *et al.*, 1972; Rhodes, 1987; Maggio *et al.*, 2002). La prolina es una molécula orgánica que se acumula en distintos organismos como plantas superiores, eubacterias, invertebrados marinos, protozoos y algas, cuando están expuestos a condiciones de estrés como sequía, salinidad, altas temperaturas, congelación, radiación ultravioleta y metales pesados (Yancey *et al.*, 1982; Delauney y Verma, 1993; Sharadi *et al.*, 1995; Kuznetsov y Sheviakova, 1997; Demir, 2000).

El papel que juega este aminoácido en la osmotolerancia ha sido ampliamente discutido (Serraj y Sinclair, 2002). En situación de estrés, se han propuesto diferentes funciones para la prolina, que puede actuar como mediador del ajuste osmótico (Handa *et al.,* 1986), como estabilizador de las estructuras celulares (Schobert y Tschesche, 1978), como eliminador de radicales libres (Smirnoff y Cumbes, 1989; Sharadi *et al.*, 1995; Hong *et al.*, 2000), como depósito de energía (Sharadi y Sharadi, 1991), como detoxificador de metales pesados (Sharma *et al.*, 1998; Rai, 2002), como señal capaz de activar respuestas específicas frente al estrés (Khedr *et al.*, 2003; Maggio *et al.*, 2003) y también ha sido descrita su importancia en la morfogénesis, como principal constituyente de las proteínas estructurales de la pared celular en plantas (Nanjo *et al.*, 1999).

La acumulación de prolina se debe fundamentalmente a síntesis *de novo* (Boggess y Stewart, 1976; Voetberg y Sharp, 1991; Roosens *et al.*, 1999), aunque se haya observado también una reducción de la tasa de su catabolismo (Rhodes *et al.*, 1986). En animales y plantas superiores, la síntesis de prolina se realiza a partir de dos sustratos: ornitina y glutamato (Bryan, 1990; Delauney y Verma, 1993). En plantas en condiciones normales la síntesis del aminoácido se produce a partir de ornitina, mediante la acción de la enzima ornitina δ-aminotransferasa (OAT), mientras que en situación de estrés se utiliza principalmente glutamato como precursor (Delauney *et al.*, 1993). El glutamato es fosforilado por la actividad kinasa de la enzima bifuncional Δ^1-pirrolin-5-carboxilato sintetasa (P5CS), que posee dos actividades enzimáticas: γ-glutamil kinasa y glutamil-γ-semialdehído deshidrogenasa, para producir γ-glutamil fosfato, el cual es reducido a glutamil-γ-semialdehído por la actividad reductasa de la P5CS. Este compuesto intermediario se cicla espontáneamente para dar Δ^1-pirrolin-5-carboxilato (P5C), el cual es reducido por la pirrolin-5-carboxilato reductasa (P5CR) a prolina. La prolina es metabolizada hasta glutamato en la mitocondria (Bogges y Koeppe, 1978) por la acción de dos enzimas prolina deshidrogenasa (ProDH), que cataliza el paso de prolina a Δ^1-pirrolin-5-carboxilato (P5C), el cual espontáneamente da lugar a

glutamil-γ-semialdehído (GSA) sobre el que actúa la enzima P5C deshidrogenasa (P5CDH), para dar finalmente glutamato (Figura 3).

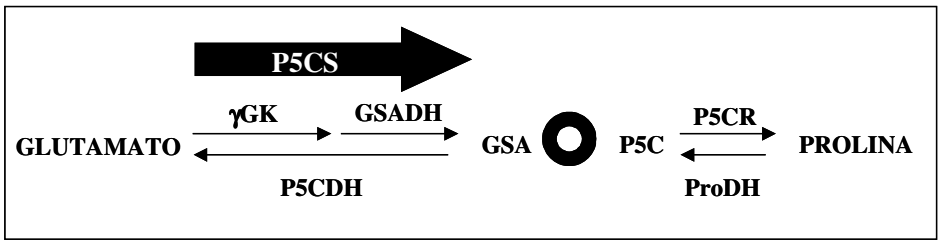


Figura 3. Metabolismo de la prolina a partir de glutamato. GSA: glutamil-γ-semialdehído; P5C: Δ^1-pirrolina-5-carboxilato; P5CS: Δ^1-pirrolina-5-carboxilato sintetasa; P5CR: P5C reductasa; γGK: γ-glutamil kinasa; GSADH: glutamil-γ-semialdehído deshidrogenasa; P5CDH: P5C deshidrogenasa; ProDH: prolina deshidrogenasa.

En condiciones de estrés hídrico los niveles de prolina en plantas están regulados principalmente a nivel transcripcional. Existe una relación positiva entre la estimulación de la biosíntesis de prolina en condiciones de salinidad y un aumento en los niveles del ARN mensajero del gen *p5cs* (Hu *et al.*, 1992; Delauney y Verma, 1993; Yoshiba *et al.*, 1995; Zhang *et al.*, 1995; Peng *et al.*, 1996; Strizhov *et al.*, 1997; Satoh *et al.*, 2002). La actividad P5CS, sujeta a inhibición por prolina, es la clave de la regulación y el paso limitante en la biosíntesis de este aminoácido. El gen que codifica la enzima P5CS es inducido por deshidratación y reprimido por rehidratación (Yoshiba *et al.*, 1997).

El catabolismo de la prolina genera una elevada energía, ya que cada molécula al oxidarse produce 30 moléculas de ATP (Atkinson, 1977). Su oxidación cede electrones a la cadena electrónica de transporte (Hare y Cress, 1997) y se ha considerado fundamental en procesos que requieren un elevado coste energético, como la germinación del polen (Zhang *et al.*, 1982). En plantas, el catabolismo de la prolina puede aportar nitrógeno amino y poder reductor a las células que se recuperan del estrés (Peng *et al.*, 1996; Verbruggen *et al.*, 1996).

2.2.2. Glicina betaína

La glicina betaína (N,N,N-trimetilglicina) es el soluto compatible predominante en la mayoría de los procariotas, y quizá el osmolito más utilizado en los reinos animal y vegetal (Rodees y Hanson, 1993; Cleland *et al.*, 2004). Es una molécula bipolar, extremadamente soluble en agua y eléctricamente neutra a pH fisiológico. Sus características moleculares le permiten interaccionar con dominios hidrófilos e hidrófobos de moléculas como enzimas o complejos proteicos.

Varias especies de plantas acumulan glicina betaína (GB) en condiciones de estrés hídrico y elevada salinidad (Wyn Jones y Storey, 1981; Hanson *et al.*, 1985; Moghaieb *et al.*, 2004), y así aumentan su tolerancia al estrés. Existe una correlación entre el nivel de GB y el grado de tolerancia al estrés (Saneoka *et al.*,

1995). Además del ajuste osmotico, se sabe que *in vitro*, estabiliza la estructura y actividad de enzimas y complejos proteicos y mantiene la integridad de las membranas (Papageorgiu y Murata, 1995). Se asume que presenta las mismas funciones *in vivo*.

En situación de estrés, la contribución de GB al equilibrio osmótico de la célula es relativamente baja (Hayashi *et al.*, 1998; Sakamoto *et al.*, 2000), por lo que el aumento de la tolerancia al estrés debe explicarse por mecanismos distintos al ajuste osmótico. En *Synechococcus*, este osmolito protege a ciertas enzimas como la Rubisco, del daño causado por estrés salino (Nomura *et al.*, 1998). Se ha descrito su función tipo chaperona molecular en *Escherichia coli* (Bourot *et al.*, 2000). Además, se ha sugerido que podría reducir la peroxidación de lípidos de membrana (Chen *et al.*, 2000). La glicina betaína también se acumula en plantas expuestas a altas y bajas temperaturas, por lo que podría desempeñar un importante papel en el mantenimiento de membranas y/o protección de complejos proteicos (Zhao *et al.*, 1992; Yang *et al.*, 1996).

En los sistemas biológicos, la síntesis de GB puede utilizar colina o glicina mediante dos rutas diferentes: deshidrogenación de colina o N-metilación de glicina (Chen y Murata, 2002). En distintas plantas, microorganismos y animales, la conversión a partir de colina, la más frecuente, tiene lugar mediante una vía con dos enzimas (Figura 4).

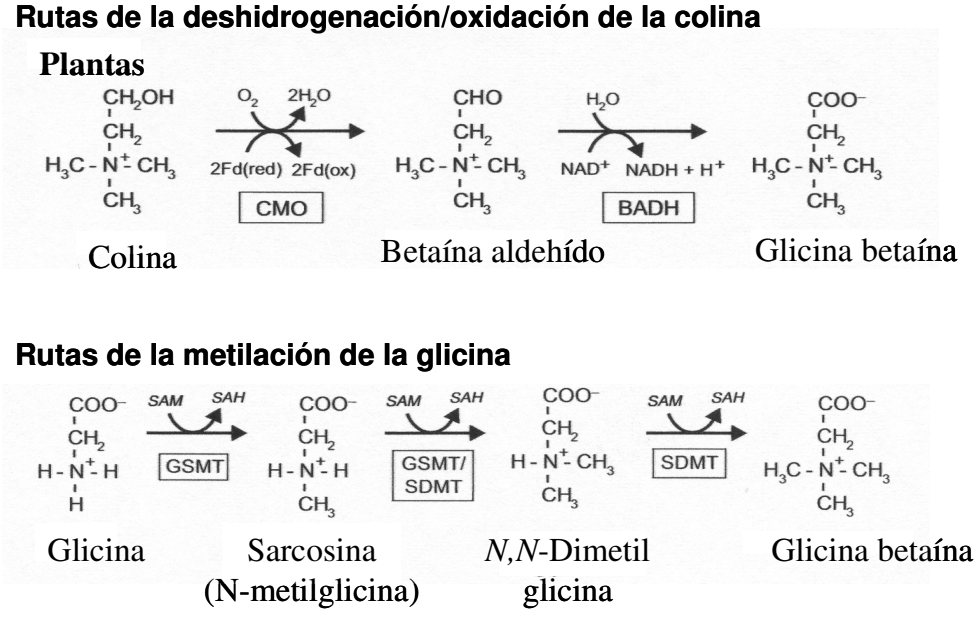


Figura 4. Principales vías de síntesis de la glicina betaína. CMO: colina monooxigenasa; BADH: betaína aldehído deshidrogenasa; Fd (red) y Fd (ox), ferredoxina en forma reducida y oxidada respectivamente; GSMT: glicin-sarcosina metiltransferasa; SDMT: sarcosin dimetiltransferasa.

En plantas, la síntesis de GB es inducida por estrés (Gorham, 1995) y las dos enzimas que participan en este proceso, colina monooxigenasa (CMO) y betaína aldehído deshidrogenasa (BADH), se encuentran localizadas en el estroma de los

cloroplastos. El paso limitante en la acumulación de este osmolito es su síntesis, más que su degradación (Rhodes y Hanson, 1993)

2.2.3. Manitol

El manitol es un hidrato de carbono de 6 átomos de carbono con grupos alcohol no cíclico que se encuentra en diversos organismos, desde las bacterias hasta las plantas. El manitol se acumula en una amplia variedad de especies de plantas en respuesta al estrés hídrico y salino (Stoop *et al.*, 1996; Patonnier *et al.*, 1999; Tang *et al.*, 2005). Además de *Apium graveolens*, otros ejemplos de plantas acumuladoras de manitol son: *Phaseolus vulgaris, Asparagus officinalis, Olea europaea*. Está presente en más de 100 especies de plantas superiores, donde puede llegar a constituir una parte importante de los hidratos de carbono solubles. El manitol se transporta vía floemática desde las hojas hacia otras partes de la planta (Keller y Matile, 1989; Davis y Loescher, 1990) y se localiza en la vacuola y en el citoplasma (Keller y Matile, 1989). Las plantas acumuladoras de manitol muestran varias ventajas. Por ejemplo, el apio, siendo una planta C_3, presenta tasas de fijación de carbono iguales a las de las plantas C_4 y son plantas que presentan un alto grado de tolerancia a la sal debido a la función del manitol como osmorregulador y soluto compatible (Tarczynski *et al.*, 1993). Otras posibles funciones del manitol incluyen el almacenamiento de carbono, la eliminación de radicales libres y, como se ha mencionado anteriormente, la osmoprotección (Smirnoff y Cumbes, 1989; Tarczynski *et al.*, 1993; Shen *et al.*, 1997). Aunque se ha propuesto que el manitol aumenta la tolerancia al estrés hídrico, fundamentalmente mediante el ajuste osmótico (Loescher *et al.*, 1992), varios trabajos en plantas transgénicas demuestran que la cantidad de manitol acumulada no es importante en términos de ajuste osmótico (Tarczynski *et al.*, 1992, 1993; Thomas *et al.*, 1995; Karakas *et al.*, 1997; Shen *et al.*, 1997).

La fructosa-6-fosfato y el NADH citoplasmáticos son utilizados por la enzima manitol-1-fosfato deshidrogenasa para formar manitol-1-fosfato y NAD^+ que a su vez originará manitol (Voegele *et al.*, 2005). Por otro lado, el manitol es fosforilado para dar manitol-1-fosfato, el cual es oxidado a fructosa-6-fosfato, que entra en la glucólisis (Figura 5).

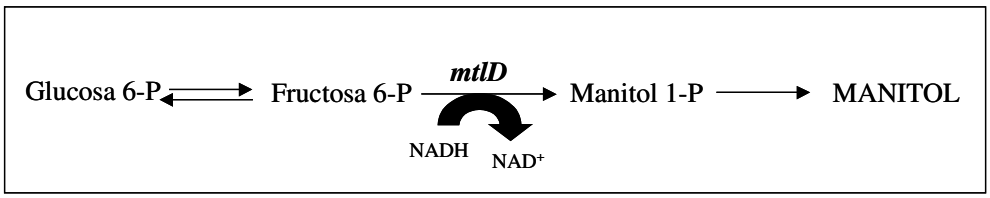


Figura 5. Metabolismo del manitol en plantas. *mtlD*: gen que codifica la enzima manitol-1-fosfato deshidrogenasa

2.2.4. Ciclitoles: pinitol y ononitol

La acumulación de los denominados ciclitoles, o hidratos de carbono con grupos alcohol cíclicos, se relaciona con la tolerancia al estrés osmótico en un elevado número de especies vegetales. Los ciclitoles pertenecen al grupo de los hidratos de carbono con grupos alcohol como el manitol, y son uno de los metabolitos que comúnmente se acumulan el organismos sometidos a estrés metabólico (Loewus y Loewus, 1980). En plantas, la mayoría de estos compuestos se sintetizan a partir del mio-inositol, hidrato de carbono ubicuo en plantas (Loewus y Dickinson, 1982). El mio-inositol es un derivado de la glucosa-6-fosfato. Puede metilarse a sequoyitol u ononitol, que son epimerizados a D-pinitol (Loewus y Loewus, 1980). El D-pinitol es el principal azúcar en la familia Pinaceae, Leguminosae y Caryophyllaceae. Su acumulación se ha observado en respuesta a la sequía como en *Cajanus cajan* (Keller y Ludlow, 1993). La acumulación de pinitol, uno de los ciclitoles más comunes, se induce por estrés en la planta halófita *Mesembryanthemum crystallinum* (Paul y Cockburn, 1989) (para revisiones ver Popp y Smirnoff, 1995), durante su adaptación a elevadas concentraciones de sal. El enzima IMT1, (*mio*-inositol O-metil transferasa), es la clave en la regulación de la acumulación de pinitol inducida por el estrés en esta planta (Vernon y Bohnert, 1992; Vernon *et al.*, 1993). El gen *Imt1*, que codifica el enzima IMT1 (Rammesmayer *et al.*, 1995) se induce transcripcionalmente en respuesta al estrés, mientras que en condiciones ambientales óptimas, no se observan ni enzima ni transcritos en la planta (Vernon y Bohnert, 1992; Vernon *et al.*, 1993).

En *M. crystallinum*, los enzimas IMT1 y ononitol epimerasa (OEP1) conducen a la síntesis de ononitol y pinitol (Figura 6), que son utilizados como osmoprotectores por la planta.

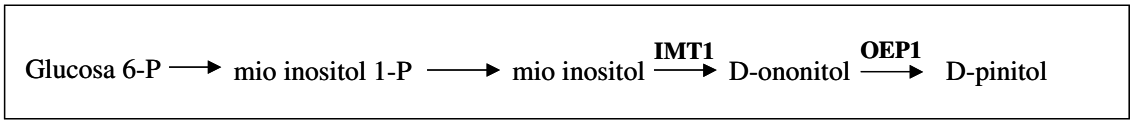


Figura 6. Metabolismo del pinitol en *Mesembryanthemum crystallinum*; IMT1: enzima *mio*-inositol O-metil transferasa; OEP1: enzima ononitol epimerasa

Los ciclitoles alcanzan concentraciones elevadas en especies de plantas tolerantes a la sal y a la sequía. Sus propiedades químicas y su naturaleza inerte los convierte en moléculas ideales para actuar como solutos compatibles en tejidos sometidos a estrés osmótico (Vernon *et al.*, 1993; McManus et al., 2000).

2.3. Acumulación de proteínas LEA

Las proteínas LEA (del inglés, Late Embryogenesis Abundant) se caracterizaron por primera vez en algodón y trigo (Cuming 1999) y son producidas en abundancia durante el desarrollo de las semillas, ocupando más del 4% de la proteína celular (Roberts *et al*. 1993). Su expresión está ligada a la tolerancia a la desecación en semillas, polen y plantas anhidrobióticas, aunque muchas proteínas LEA son inducidas por frío o estrés osmótico, por ácido abscísico exógeno o incluso son expresadas constitutivamente como es el caso de la *dhnX* de *Arabidopsis thaliana* (Welin *et al*. 1994). Las investigaciones indican un posible papel de las proteínas LEA en la tolerancia a la deshidratación, probablemente a través del mantenimiento de la estructura de proteínas o membranas, secuestro de iones, captación de agua y función como chaperonas moleculares (Close, 1997; Brownie *et al*., 2002).

Los genes de las proteínas LEA se han identificado en muchas especies vegetales, y finalmente se han definido seis grupos diferentes de proteínas LEA basándose en su patrón de expresión y secuencia; las principales categorías son Grupo 1, Grupo 2 y Grupo 3 (Bray, 1993; Cuming, 1999; Wise, 2003). El Grupo 1 de proteínas LEA, del cual la proteína Em de trigo es la secuencia tipo, está a su vez dividido en dos superfamilias por Wise (2003) y solo se encuentra en plantas. No tienen estructura en solución (McCubbin *et al*. 1985), pero contienen un motivo conservado de 20 aminoácidos, principalmente en una sola copia (Cuming 1999). El Grupo 2 de proteínas LEA, que según Wise (2003) engloba tres superfamilias, son también conocidas como "dehidrinas" (Close, 1996), y están presentes principalmente en plantas, incluyendo algas. El Grupo 2 de proteínas se caracteriza por tener más de tres secuencias conocidas como **dominio K** (rico en lisina y cuya secuencia es EKKGIMDKIKEKLPG), **dominio Y** (DEYGNP) y el **segmento S** (fragmento de poli-serina). El dominio Y tiene una secuencia aminoacídica importante relacionada con una porción del sitio de unión a las chaperonas de plantas y bacterias (Martin *et al*., 1993) y por otro lado, en RAB17 de maíz y TAS14 de tabaco, se ha demostrado que los residuos de Ser del segmento S pueden fosforilarse, lo cual está relacionado con la unión a péptidos señal de localización nuclear y por tanto, con el transporte nuclear (Goday *et al*., 1994; Godoy *et al*. 1994). Estas proteínas del Grupo 2 también presentan una estructura poco definida aunque algunas muestran un contenido de α-hélices (Ceccardi *et al*. 1994; Close, 1996; Lisse *et al*. 1996). El Grupo 3 de proteínas LEA comprende dos superfamilias (Wise 2003) que se caracterizan por tener una secuencia consenso definida como ΦΦE/QXΦLE/QLΦXE/D/Q (donde Φ representa un residuo hidrofóbico) (Dure, 2001). Este grupo presenta un interés especial debido al descubrimiento de homólogos en otros organismos como los nematodos *Caenorhabditis elegants, Steinernema feltiae* y en los procariotas *Bacillus subtilis* y *Haemophilus influenzae* (Solomon *et al*. 2000; Dure 2001; Browne *et al*. 2002).

Basándose en el patrón de expresión, análisis de localización de la proteína, distribución de residuos de aminoácidos a lo largo de polipéptido y análisis de plantas transgénicas, se ha propuesto que las proteínas LEA puedan tener una función protectora en la supervivencia de la planta bajo condiciones de estrés

osmótico. Las proteínas LEA interactúan con otras y estabilizan así los constituyentes subcelulares como membranas o ácidos nucleicos (Imai *et al.*, 1996; Xu *et al.*, 1996; Arenas-Mena *et al.*, 1999). En muchos estudios aparece la implicación de estas proteínas en respuestas a la deshidratación en plantas frente a un rango de estímulos ambientales. Algunas evidencias apuntan a su actuación como chaperonas o a la actividad como detergentes que es iniciada por interacciones hidrofóbicas y que conducen a fuerzas de hidratación. Las múltiples dianas de las proteínas LEA (eucromatina, citosol, citoesqueleto...) sugieren que las consecuencias directas de dichas proteínas son bioquímicamente diversas (Close, 1996).

La correlación entre expresión de los genes que codifican proteínas LEA o bien la acumulación de proteínas LEA y la tolerancia al estrés en gran número de plantas confirma la idea de que las proteínas LEA tienen una función en la tolerancia al estrés. Por ejemplo, en muchas semillas deshidratadas de trigo, la acumulación de altos niveles de proteínas LEA del Grupo 3, se relacionó con tolerancia a la deshidratación de los tejidos (Ried y Walker-Simmons, 1993). Muchos estudios realizados con variedades de arroz (*Oriza sativa* L.) mostraron que los niveles de proteínas LEA del Grupo 2 y del Grupo 3 en raíces eran significativamente más elevados en variedades resistentes a la sal comparado con variedades sensibles (Moons *et al.*, 1995). En un trabajo de Hong *et al.*, (1992) se vio que la expresión del gen HVA1 es rápidamente inducida en semillas jóvenes por ABA y por varias condiciones de estrés como deshidratación, sal y temperatura extrema. En relación con el mismo gen, se ha comprobado posteriormente que la acumulación de la proteína HVA1 de cebada en los tejidos vegetativos de plantas transgénicas de arroz, confiere un aumento de tolerancia al déficit hídrico y estrés salino y que esta tolerancia al estrés se corresponde con el nivel de acumulación de la proteína HVA1 (Xu *et al.*, 1996; Babu *et al.*, 2004). Estos resultados no solo demuestran un papel para la proteína LEA de cebada en la protección frente al estrés, sino que también sugieren un gran potencial no aprovechado aún de los genes que codifican LEA para la ingeniería genética de la tolerancia al estrés.

2.4. Regulación de la acumulación de aquaporinas

Otro mecanismo de protección frente al estrés hídrico consiste en regular el movimiento del agua, tanto a nivel intracelular como a través de los tejidos y órganos de la planta. Las membranas de la planta juegan un papel crítico en la homeostasis celular y en la señalización, nutrición y respuesta a los estreses. Durante los años 90, se han investigado las bases moleculares y celulares del transporte de membrana pero el progreso ha sido particularmente importante en el campo de las relaciones hídricas y los mecanismos de transporte de agua a través de las membranas celulares. Así, se ha llegado a una caracterización molecular y funcional de una nueva clase de proteínas de membrana, que forman la superfamilia de Proteínas Intrínsecas de Membrana (MIP, del inglés Membrane Intrinsic

Proteins). Éstas constituyen canales de agua que facilitan el flujo de agua siguiendo un gradiente de potencial hídrico (Maurel, 1997; Maurel y Chrispeels, 2001).

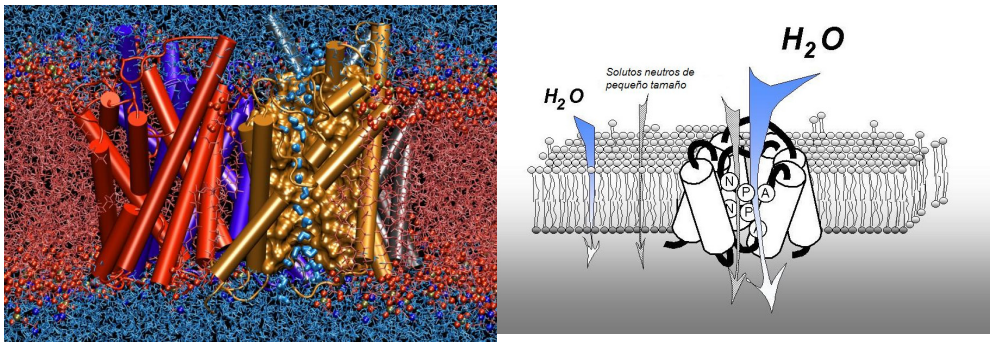


Figura 7. Imagen tridimensional de una aquaporina (izquierda). Esquema de la localización, estructura y función de las aquaporinas (derecha).

Las aquaporinas muestran una estructura típicamente conservada con seis dominios transmembrana unidos por tres extra- y dos bucles intracelulares, un extremo N-terminal y otro C-terminal en la cara del citosol (Fujiyoshi *et al.*, 2002) (Figura 8)

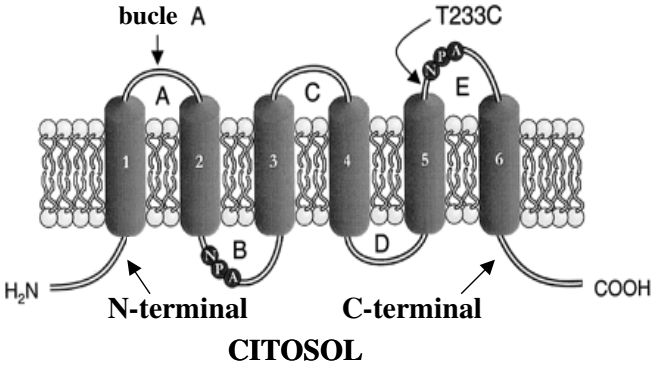


Figura 8. Representación esquemática de la estructura de un monómero de aquaporina, con sus seis dominios transmembrana (1-6) y cinco bucles que los conectan (A-E). Se muestran los motivos NPA altamente conservados.

Los motivos aminoacídicos conservados, como los dos Asn-Pro-Ala también definen una característica de la secuencia de la aquaporina. Normalmente las aquaporinas constituyen tetrámeros donde cada monómero forma un poro independiente.

En plantas, las aquaporinas se presentan como diversas isoformas. Por ejemplo, el genoma de *Arabidopsis thaliana* codifica 35 aquaporinas homólogas, y se predice una diversidad similar en otras especies vegetales (Chaumont *et al.*, 2001; Johanson *et al.*, 2001; Quigley *et al.*, 2001). La familia de las aquaporinas puede

subdividirse en cuatro clases homólogas, con distinta localización. Las aquaporinas más abundantes de la membrana plasmática y vacuola pertenecen a las clases proteína intrínseca de membrana plasmática (PIP del inglés, plasma intrinsic protein,) y proteína intrínseca del tonoplasto (TIP del inglés, tonoplast intrinsic protein), respectivamente. La clase PIP es a su vez dividida en dos grupos homólogos, PIP1 y PIP2, que en *Arabidopsis thaliana* cuenta con cinco y ocho miembros, respectivamente. Otra aquaporina que se expresa en la membrana peribacteroidal de los nódulos de las raíces fijadoras de nitrógeno recibe el nombre de nodulina 26 de soja. Esta aquaporina y sus homólogos cercanos forman el grupo de proteínas intrínsecas como la nodulina 26 (NIPs, Nodulin-26-like intrinsic protein). Las NIPs también están presentes en especies de plantas no leguminosas donde hasta el momento se desconoce su localización. La cuarta clase de aquaporinas de plantas engloba a las proteínas intrínsecas pequeñas básicas (SIPs del inglés, small basic intrinsic protein). Su función y localización son actualmente desconocidas (Luu y Maurel, 2005).

Se ha estudiado la función y regulación de PIPs. Estas aquaporinas parecen desempeñar una función específica e importante en el control del transporte transcelular del agua. Son expresadas abundantemente en raíces donde intervienen en la captación de agua del suelo (Javot y Maurel, 2002). Las TIPs median en el intercambio de agua entre el compartimento del citosol y el vacuolar y pueden jugar un papel fundamental en la osmorregulación celular (Maurel *et al.*, 1997; Tyerman *et al.*, 1999). La nodulina-26 transporta pequeñas moléculas sin carga como el glicerol o la urea y se ha sugerido que tenga un papel en el transporte de amonio (Dean *et al.*, 1999; Niemietz y Tyerman 2000). Así, esta aquaporina puede intervenir en funciones de intercambio entre las células de la raíz infectada y el endosimbionte, y posiblemente contribuya a la osmorregulación del espacio peribacteroidal. Recientemente, se han hecho estudios que muestran la contribución de PIPs a la difusión de CO_2 por los tejidos (Terashima y Ono, 2002; Uehlein *et al.* 2003; Hanba *et al.*, 2004).

Se ha sugerido que cuando las aquaporinas de vacuola y membrana plasmática actúan conjuntamente, son responsables de la osmorregulación del citosol, necesaria para mantener los procesos metabólicos (Kjelbom *et al.* 1999). Sin embargo, los estudios de inhibición llevados a cabo *in vivo* y en mutantes antisentido han indicado que las aquaporinas son también importantes para el flujo de agua en plantas (Kjelbom *et al.* 1999). La elevada expresión de genes que codifican aquaporinas en tejidos implicados en transporte hídrico sugiere un papel en el transporte de agua transcelular (Barrieu *et al.* 1998)

En los últimos años, se han propuesto varias hipótesis sobre los mecanismos de regulación de las aquaporinas de plantas a nivel celular, tisular, subcelular y molecular. Probablemente, las aquaporinas interactúan con otras proteínas "acompañantes" para llevar a cabo una modificación post-translacional, actuaciones subcelulares o incluso degradación pero aún no han sido identificadas estas proteínas. Mientras que está comenzando a emerger el papel funcional de glicosilación y fosforilación, las aquaporinas de plantas, probablemente experimenten otros tipos de maduraciones post-translacionales como

ubiquitinación y/o modificación controlada de proteínas, e incluso nitrosilación (Shey *et al.*, 1999; Hess *et al.*, 2001; Leitch *et al.*, 2001).

Actualmente se sabe que el estrés hídrico actúa sobre la función de la aquaporina a nivel de transcripción, relocalización de proteínas y entrada mediante fosforilación reversible o mediante efectos directos sobre los gradientes osmóticos o hidrostáticos (Luu y Maurel, 2005) (Figura 9). Por ello, conocer cómo las plantas integran estos mecanismos en el tiempo y espacio y ajustan el transporte hídrico y las propiedades del transporte de solutos de sus membranas, constituye actualmente un verdadero desafío.

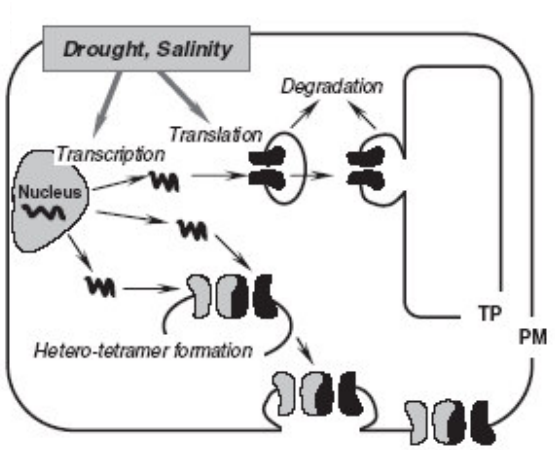


Figura 9. Control de la transcripción y abundancia de proteínas. Se sabe que la sequía y salinidad, como otros estímulos ambientales, actúan sobre la transcripción de genes de aquaporinas y posiblemente interfieran con la translación y degradación de éstas, determinando así la abundancia de proteínas. La formación de PIP-heterotetrámeros se demostró en oocitos de *Xenopus leavis* (Fetter *et al*., 2004) pero todavía es una hipótesis en células vegetales. Este mecanismo puede favorecer la transferencia de homólogos PIP1 a la membrana plasmática (PM) (Adaptado de Luu y Maurel, 2005)

2.5. Inducción de proteínas antioxidantes

Uno de los cambios bioquímicos que tienen lugar cuando las plantas están sometidas a estreses ambientales es la producción de especies reactivas del oxígeno (ERO) (Dat *et al.*, 2000). El peróxido de hidrógeno (H_2O_2) y el radical superóxido (O_2^-) se forman en la cadena de transporte de electrones de cloroplastos y mitocondrias, y mediante muchas oxidasas dependientes de flavinas, incluyendo la glicolato oxidasa de peroxisomas (Del Río *et al.*, 1992). El peróxido de hidrógeno y el superóxido también son generados por mecanismos no enzimáticos como la oxidación catalizada por Fe del NAD(P)H, glutation reducido (GSH) y ascorbato (ASC). Ambas especies no son reactivas en condiciones fisiológicas óptimas. Sin embargo, metales como el Fe^{3+} o el Cu^{2+} mediante la reacción de Fenton, catalizan la conversión en una de las moléculas más reactivas conocidas en la naturaleza, el radical hidroxilo (OH^-), que origina daño celular por peroxidación de membranas, desnaturalización de proteínas y daño en el ADN. El peróxido de

hidrógeno puede inactivar varios enzimas del Ciclo de Calvin (Asada y Takahashi, 1987; Noctor y Foyer, 1998). Otra especie altamente reactiva es el oxígeno singlete ($^1\Delta_g O_2$), que es formado principalmente en reacciones fotodinámicas en el cloroplasto (Asada y Takahshi 1987; Elstner, 1987).

Para evitar la producción de estas moléculas reactivas, las plantas han desarrollado un sistema de secuestro efectivo que implica moléculas antioxidantes como carotenoides, ascorbato, glutation y tocoferol, además de enzimas antioxidantes como superóxido dismutasa (SOD; EC 1.15.1.1.), catalasa (CAT; EC 1.11.1.6.), ascorbato peroxidasa (APX; EC 1.11.1.11.) y glutation reductasa (GR; EC 1.8.5.1.).

Según Smirnoff (1993), el "pull" de enzimas antioxidantes puede dividirse en dos categorías: sistemas que reaccionan con formas activas del oxígeno y las mantienen a bajo nivel (SOD, CAT o APX), y sistemas que regeneran antioxidantes oxidados (glutation GSH, GR, mono- y dehidroascorbato reductasas) (Figura 10)

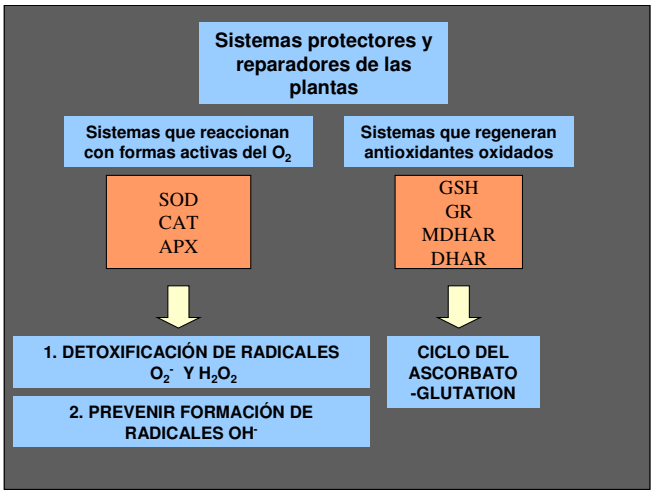


Figura 10. Clasificación de los sistemas protectores y reparadores en plantas frente al daño oxidativo según Smirnoff (1993)

El primer grupo de enzimas está implicado en la detoxificación de radicales O_2^- y H_2O_2, previniendo la formación de radicales OH^-. La GR y el GSH, son componentes importantes del ciclo ascorbato-glutation, responsable de la eliminación del H_2O_2 en diferentes compartimentos celulares (Dalton, 1995; Jiménez *et al.*, 1997).

Las especies reactivas del oxígeno están implicadas en el daño molecular observado en plantas expuestas a gran variedad de condiciones adversas incluyendo sequía, salinidad, alta radiación, luz ultravioleta, baja temperatura, contaminantes del aire (especialmente ozono), tratamiento con herbicida y ataque por patógenos (Scandalios *et al.*, 1997; Noctor y Foyer 1998).

La sequía es uno de los problemas que afectan al crecimiento y productividad de la planta provocando estrés osmótico y toxicidad de iones dando lugar a un estrés oxidativo. Se estima que el 10-20% de los electrones que pasan por el fotosistema I reducen el O_2 a H_2O_2 (Asada 1999) y que el 2% del O_2 consumido por la mitocondria es usado para generar H_2O_2 (Scandalios *et al.*, 1997). Por ello, las plantas y otros organismos aeróbicos dependen de una defensa antioxidante para evitar su propia destrucción.

Superóxido dismutasa (EC 1.15.1.1.)

La familia SOD está compuesta por metaloproteínas que catalizan la dismutación de radicales O_2^- en O_2 y H_2O_2. Se conocen tres clases de SODs en plantas dependiendo del cofactor usado como sitio activo (Mn, Fe o Cu-Zn). Así, encontramos MnSODs y FeSODs estructuralmente relacionadas pero de localización distinta. La MnSOD se encuentra en la mitocondria mientras que la FeSOD se localiza en el cloroplasto. La CuZnSOD no está relacionada estructuralmente con las anteriores y la podemos encontrar en el citosol y cloroplasto. Las tres isoformas muestran propiedades moleculares distintas incluyendo una sensibilidad diferencial a los inhibidores. La SOD tiene un importante papel en la protección frente al estrés oxidativo (Santos *et al.*, 2000; Moran *et al.*, 2003).

Catalasa (EC 1.11.1.6.)

La catalasa de plantas está constituida por homoproteínas tetraméricas que existen en múltiples isoformas codificadas por genes nucleares. Están localizadas mayoritariamente en peroxisomas y glioxisomas aunque una isoforma específica, la Cat3, está presente en mitocondria de maíz (Scandalios *et al.*, 1997). La ausencia de CAT en el cloroplasto descarta que tenga un papel en la protección de los enzimas regulados por tiol del Ciclo de Calvin. Su función es la de convertir el H_2O_2 en agua y oxígeno molecular en los peroxisomas.

Ciclo del ascorbato-glutation

El ciclo del ascorbato-glutation es una de las principales defensas antioxidantes en plantas y su función reconocida es su intervención en la eliminación de H_2O_2 en el cloroplasto (Figura 11). Sin embargo, todos los componentes de esta ruta están también presentes en la mitocondria y peroxisomas de hojas (Jiménez *et al.*, 1997) y en el citosol de nódulos de leguminosas (Dalton *et al.* 1986, 1992; Dalton, 1995).

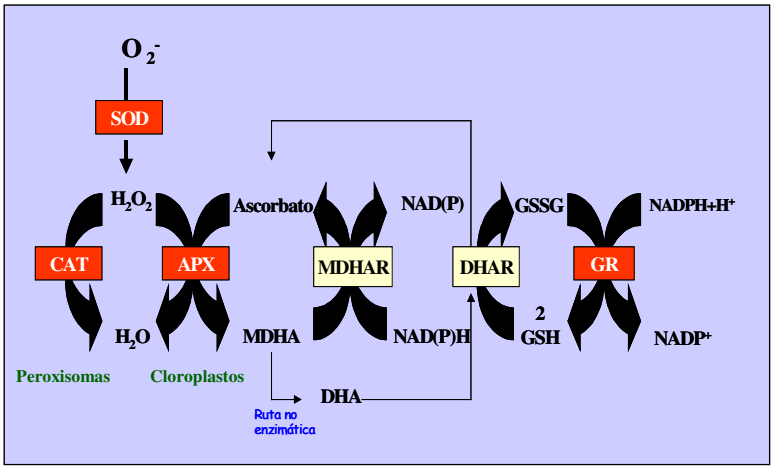


Figura 11. Ciclo del ascorbato-glutation (SOD, superóxido dismutasa; CAT, catalasa; APX, ascorbato peroxidasa; MDHAR, monodehidroascorbato reductasa; DHAR, dehidroascorbato reductasa; GR, glutation reductasa; MDHA, monodehidroascorbato; GSSG, glutation oxidado; GSH, glutation reducido)

El ciclo implica la acción concertada de cuatro enzimas: ascorbato peroxidasa (APX), dehidroascorbato reductasa (DHAR), monodehidroascorbato reductasa (MDHAR) y glutation reductasa (GR), y requiere un continuo aporte de ascorbato, tioles y nucleótidos de piridina reducidos (Dalton *et al.*, 1986, 1992).

La primera reacción de la ruta es catalizada por la **ascorbato peroxidasa** (EC 1.11.1.11.). La APX es la peroxidasa más importante en la detoxificación del H_2O_2 en cloroplastos (Noctor y Foyer 1998). Este enzima cataliza la reducción del H_2O_2 a agua usando el poder reductor del ascorbato, y el MDHA y DHA resultantes, son reducidos de nuevo a ascorbato mediante la MDHAR y DHAR más GR, respectivamente (Iturbe-Ormaetxe *et al.*, 2001).

Las preoxidadas están asociadas con procesos bioquímicos y fisiológicos como el crecimiento, formación de las células, desarrollo del fruto, biosíntesis de etileno y cómo no, en la respuesta a varios estreses (Matamoros *et al.*, 2003)

La **glutation reductasa** es el enzima que regenera el GSH oxidado en su forma reducida en el Ciclo del ASC-GSH. Kranner (2002) relacionó la cantidad de GSH reducido con diferentes grados de tolerancia a la desecación en líquenes. Se ha propuesto que la GR tiene una importante función en la protección de las plantas frente a diversas formas de estrés (Aono *et al.*, 1995). La mejora en la actividad GR se ha asociado con incrementos en el contenido de ASC (Foyer *et al.*, 1995), mejor protección del "pull" de ascorbato y glutation en el estrés frente al paraquat (Foyer *et al.*, 1991, 1995), disminución de la sensibilidad a la fotoinhibición (Foyer *et al.*, 1995) y daño foliar mitigado durante la exposición al paraquat (Aono *et al.*, 1993).

Antioxidantes y senescencia nodular

La fijación de nitrógeno en las leguminosas es particularmente sensible a las perturbaciones ambientales, incluyendo defoliación, estrés hídrico o salino, oscuridad continuada y exceso de nitratos (Vance *et al.*, 1979; Witty *et al.*, 1986; Layzell *et al.*, 1990; Lorenzo *et al.*, 1994; Gorgocena *et al.*, 1995, 1997; Matamoros *et al.*, 1999). En la mayoría de los tipos de estrés, la disminución inicial de la actividad nitrogenasa está asociada con una disminución en la concentración de O_2. La prolongación del estrés induce senescencia nodular prematura, que comparte algunas características con la senescencia natural (envejecimiento del nódulo), tales como la pérdida de la fijación de N_2, incremento en las actividades líticas y la formación de pigmentos verdes a partir de la leghemoglobina (Lb) (Pfeiffer *et al.*, 1983; Sarath *et al.*, 1986). Esta senescencia inducida por estrés se ha relacionado con la producción de oxidantes y la reducción de defensas antioxidantes (Escuredo *et al.*, 1996; Gorgocena *et al.*, 1997; Jebara *et al.*, 2005).

Los nódulos tienen elevados niveles de respiración debido a la gran demanda de energía de la fijación de N_2 que origina un gran flujo de O_2 en el nódulo y con ello, un alto riesgo de formación de especies reactivas del oxígeno (Dalton, 1995). Entre las principales fuentes de formación de ERO encontramos:

- La leghemoglobina, que probablemente sea la más importante. Es una hemoproteína como la mioglobina localizada en el citosol de células infectadas por rizobios. Facilita el transporte de O_2 a los bacteroides (células donde se lleva a cabo la fijación de N_2) con un flujo bajo pero constante, de forma que el O_2 prevenga la inactivación de la nitrogenasa (Appleby, 1984).
- Proteínas lábiles del O_2. Son proteínas capaces de reaccionar con O_2 para generar ERO. Destaca la dinitrogenasa (MoFe) y especialmente la dinitrogenasa reductasa (Fe) y el cofactor FeMo de la nitrogenasa, que son irreversiblemente dañadas por el O_2.
- Actividades enzimáticas naturales del nódulo. La xantin-oxidasa (EC 1.1.3.22) y la uricasa (EC 1.7.3.3.) son los primeros enzimas de la ruta de degradación de las purinas y producen O_2^- y H_2O_2, respectivamente. La lipooxigenasa (EC 1.13.11.12.) es otro enzima que genera ERO. Cataliza la oxidación de ácidos grasos poliinsaturados mediante el O_2 y produce hidroperóxidos y radicales O_2^- (Lynch y Thompson 1984)
- Fe catalítico. Los nódulos son extremadamente ricos en Fe como un constituyente esencial de la nitrogenasa, ferritina y Lb (Ragland y Theil 1993). El Fe libre y la mayoría de las formas queladas del Fe (incluyendo el Fe en hemo) catalizan la descomposición del H_2O_2 en OH^- y promueven la peroxidación lipídica.

Durante la senescencia natural de los nódulos, algunos antioxidantes (GSH, hGSH, catalasa) y no otros (APX, ASC, α-tocoferol), disminuyen (Dalton *et al.*, 1986, Swaraj *et al.*, 1995, Evans *et al.*, 1999; Matamoros *et al.*, 1999). En la mayoría de los casos, asociado a la inhibición de la nitrogenasa y la degradación de

la Lb, se ha visto una disminución en las principales actividades implicadas en la eliminación de H_2O_2, tales como APX, catalasa, tioles y contenido de ASC.

2.6. Proteínas 14-3-3

Las proteínas 14-3-3 fueron originariamente identificadas y nombradas como parte de una familia de proteínas ácidas solubles de cerebro bovino (Moore y Pérez, 1967). En un principio fueron designadas numéricamente en base al fraccionamiento por columna y movilidad electroforética pero actualmente son conocidas con letras griegas (Figura 12).

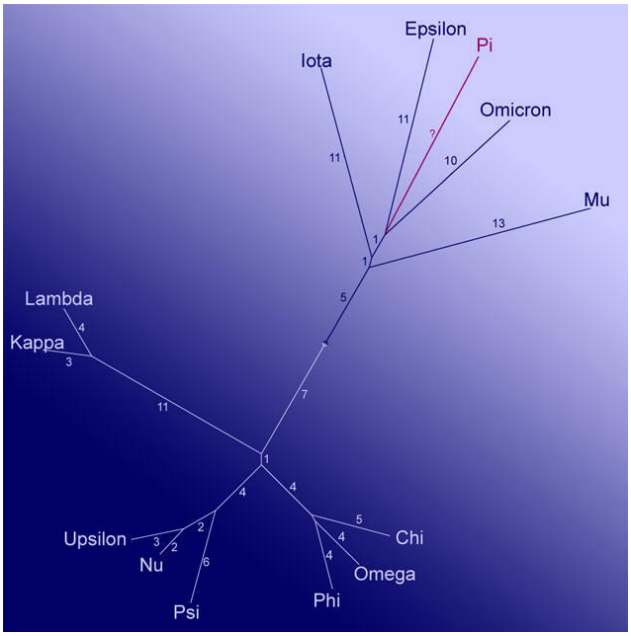


Figura 12. Filogenia y nomenclatura de las proteínas 14-3-3.

Son proteínas de aproximadamente 30 kDa y actualmente se sabe que están presentes en todos los eucariotas. Se ha demostrado tanto *in vivo* como *in vitro*, la formación de homo- y heterodímeros mediante las regiones N-terminal de distintas isoformas de 14-3-3 (Jones *et al.*, 1995; Wu *et al.* 1997), sin embargo, no hay un acuerdo en cuanto a la conservación de la secuencia entre todos los isotipos de 14-3-3, incluso entre isoformas de animales y plantas. Cada secuencia de la proteína 14-3-3 puede ser dividida en tres regiones: un extremo amino variable, la región central conservada y un extremo carboxilo variable (Figura 13).

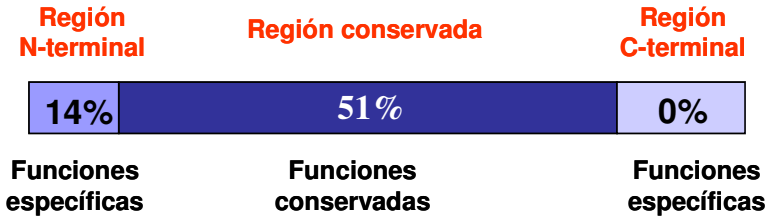


Figura 13. Estructura fundamental de las 14-3-3 que muestra las distintas regiones de la proteína. Los porcentajes indican el grado de conservación de la secuencia de aminoácidos entre todas las isoformas (Adaptado de Chung *et al*., 1999).

Las proteínas 14-3-3 son dímeros en los que cada monómero consiste en algo más de nueve α-hélices alineadas de forma antiparalela, creando un surco lo suficientemente grande para albergar una porción de péptido de la proteína diana (Figura 14). Estos datos apoyan la idea de que un solo dímero de 14-3-3 puede unirse simultáneamente a dos dianas o bien a dos áreas de la misma diana (Ferl, 1996; Yaffe *et al*., 1997), lo que conduce a un posible modelo de acción donde las 14-3-3 tienen un papel importante en la construcción de proteínas diana (Tzivion *et al*., 2001) y en la modificación de la estructura de proteínas diana individuales (Yaffe, 2002)

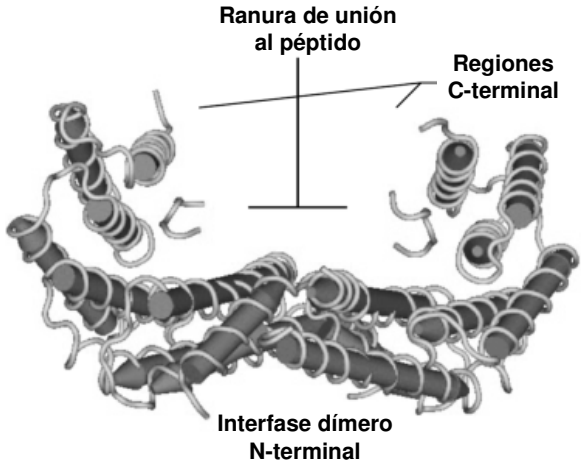


Figura 14. Estructura cristalina de un dímero de 14-3-3. La estructura central del dímero está compuesta por hélices antiparalelas. Cada monómero del dímero crea un surco que forma la interfase donde se une a la diana. (Adaptado de Ferl, 2004).

Desde el descubrimiento de estas proteínas, se ha investigado de una forma intensa su importancia y función. Las proteínas 14-3-3 están implicadas en división celular, apoptosis, rutas de señalización, generalmente funcionando como adaptadores, chaperonas, activadores y represores (Palmgren *et al*., 1998). Se unen a proteínas diana que incluyen proteínas implicadas en el metabolismo, transducción de señales, función de la cromatina, transporte de iones y tráfico de vesículas. También es bien sabido que la nitrato reductasa (NR) y la H^+-ATPasa de

la membrana plasmática son inhibidas y activadas por la 14-3-3, respectivamente. Estas proteínas no están por sí mismas implicadas en la señalización, pero su interacción con las 14-3-3 representa el paso final en la cascada de señalización dirigida a su regulación. La nitrato reductasa, por ejemplo, es inactivada por la 14-3-3 seguida de una fosforilación por la proteín kinasa en respuesta a la luz (Huber *et al.*, 2002; Comparot *et al.*, 2003) (Figura 15). Las CDPKs (protein-kinasas dependientes de calcio) constituyen una única familia de kinasas de plantas que son definidas por el dominio C-terminal regulado como la calmodulina. Se ha visto que están implicadas mediante interacción con las 14-3-3 en señalización del estrés. De hecho, el calcio es un importante segundo mensajero en múltiples rutas de señales de transducción en plantas y que aumentan momentáneamente la concentración en el citosol en respuesta a distintos estreses abióticos, incluyendo sequía, frío o estrés salino (Knight *et al.*, 1991, 1997). Por otro lado, también se ha demostrado que el sitio de unión de la 14-3-3 a la H^+-ATPasa de las células del estoma es fosforilado en respuesta a la luz azul como un paso final en la ruta de señalización al incrementarse la apertura estomática (Kinoshita y Shimazaki, 1999).

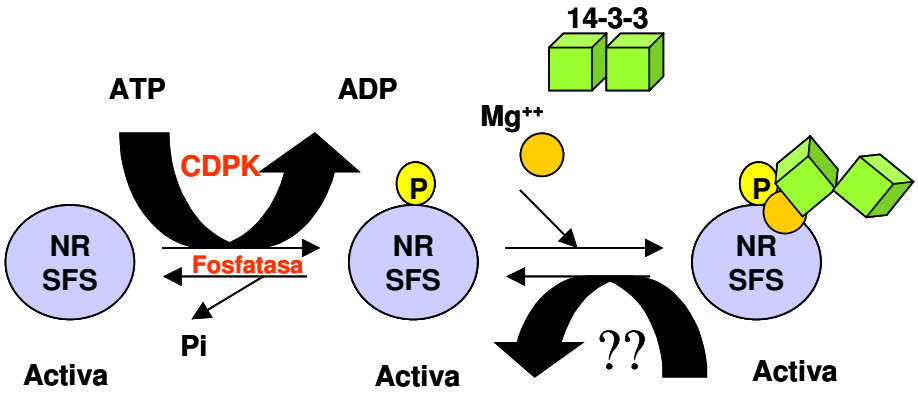


Figura 15. Regulación metabólica de la NR (nitrato reductasa) y SFS (sacarosa fosfato sintasa). Ambas están reguladas en dos pasos que implican la fosforilación del enzima diana seguido de la unión de la 14-3-3. El primer paso es la fosforilación del enzima por una kinasa dependiente de Ca^{++} (CDPK) . Este paso solo no inactiva la diana y puede ser revertido por la actividad de la fosfatasa. En el segundo paso, la unión de Mg^{++} a la 14-3-3 origina un cambio estructural que permite la unión a la proteína fosforilada y la inactivación del proceso. (Adaptado de Chung *et al.*, 1999)

En plantas, la H^+-ATPasa de la membrana tiene un papel esencial en el mantenimiento del turgor. Por hidrólisis del ATP, la H^+-ATPasa exporta protones para crear un gradiente electroquímico a través de la membrana plasmática, lo cual luego es usado por las células como fuerza conductora para el transporte de iones y metabolitos. La actividad de la bomba de protones de la membrana plasmática no es constante y aumenta bajo estrés osmótico (Curti *et al.*, 1993).

A las 14-3-3 se le ha asignado un papel en las respuestas de defensa frente a patógenos (Brandt *et al.*, 1992; Roberts y De Bruxelles, 2002), pero también hay evidencias de una activación de la H^+-ATPasa de membrana plasmática mediada por

14-3-3 en respuesta a estreses abióticos como el estrés osmótico y las bajas temperaturas (Chelysheva *et al.*, 1999; Kerkeb *et al.*, 2002). Hay estudios que correlacionan un incremento en la cantidad de complejos H^+-ATPasa –14-3-3, en la actividad de la bomba de protones de la H^+-ATPasa y en la cantidad de sitios de unión de la fusicocina con una disminución en el turgor de células de remolacha azucarera cultivadas en suspensión y sometidas a estrés por frío (Chelysheva *et al.*, 1999) o estrés osmótico (Babakov *et al.*, 2000) (Figura 16).

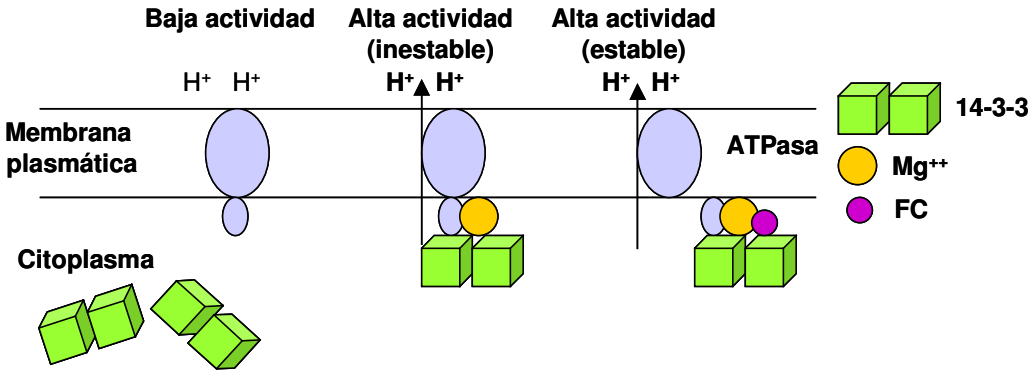


Figura 16. Regulación de la H^+-ATPasa de membrana plasmática por las proteínas 14-3-3 y la fusicocina (FC). La unión de la proteína 14-3-3 al dominio C-terminal de la H^+-ATPasa es dependiente de Mg^{++}. El complejo 14-3-3- H^+-ATPasa también origina un sitio de unión a la FC y por tanto la unión de ésta estabiliza el complejo, desplazando el dominio C-terminal y activando completamente a la H^+-ATPasa. (Adaptado de Chung *et al*., 1999).

Desde el punto de vista de los hongos, la información existente sobre proteínas 14-3-3 es bastante menor. Aunque la mayoría de los datos mostrados proceden de estudios en humanos, mamíferos y plantas, la estructura y función de las proteínas 14-3-3 de levaduras parece estar también muy conservada. En una revisión de Van Hemert *et al.*, 2001 se discuten las diversas funciones encontradas en las 14-3-3 de levaduras. Se ha propuesto que, al igual que las proteínas 14-3-3 de eucariotas superiores, las proteínas Bmh son reguladoras en una variedad de rutas de señalización celular en *Saccharomyces cerevisiae* (Van Hemert *et al.*, 2001). El hongo *S. cerevisiae* tiene dos genes, *bmh1* y *bmh2*, que codifican proteínas 14-3-3 (Van Heusden *et al.*, 1992, 1995; Gelperin *et al.*, 1995; Van Hemert *et al.*, 2001). Una mutación *bmh1 bmh2* es letal en la mayoría de las razas de laboratorio, y dicha mutación se puede complementar al final por cuatro isoformas de *Arabidopsis* (Van Heusden *et al.*, 1996). Como en los eucariotas superiores, las proteínas 14-3-3 de *S. cerevisiae* están implicadas en muchos procesos celulares, y se han identificado muchas proteínas de unión (Van Hemert *et al.*, 2001).

2.7. Proteínas BiP

El lumen del retículo endoplasmático (RE) es un ambiente especializado que promueve la síntesis de un "set" de proteínas secretoras destinadas al compartimento vacuolar o la matriz extracelular (Vitale *et al.*, 1993). Entre la familia de proteínas del RE identificadas en plantas, la proteína de unión al lumen BiP, (del inglés Binding Protein) es una de las más caracterizadas (Denecke 1996). BiP es una chaperona ubicua y forma parte de la familia de proteínas hsp70 reguladas por estrés (Munro y Pelham, 1986).

A diferencia de otros miembros de esta familia, BiP es sintetizada con una secuencia señal N-terminal para la translocación en el RE, y una secuencia C-terminal que determina su localización fuera del RE (Munro y Pelham, 1986, 1987).

Las funciones de la proteína BiP son varias, pero se ha comprobado que puede prevenir la secreción de proteínas que todavía no han adquirido su conformación madura (Bole *et al.*, 1986; Gething *et al.*, 1986), promover la construcción y ensamblaje de proteínas (Gething *et al.*, 1986) o disolver agregados de proteínas que se han originado bajo condiciones normales o condiciones de estrés en el RE (Munro y Pelham, 1986). En estudios in vitro usando péptidos sintéticos se ha demostrado que BiP tiene una gran afinidad por polipéptidos con un alto grado de hidrofobicidad (Blond-Elguindi *et al.*, 1993).

En algunas plantas como tabaco, maíz y soja, BiP está codificada por una familia multigénica (Denecke *et al.*, 1991; Fontes *et al.*, 1991; Kalinski *et al.*, 1995), situación que solo parece ocurrir en plantas. Sin embargo, encontramos excepciones en espinaca (Anderson *et al.*, 1994) y *Arabidopsis* (Koizumi, 1996) donde no se ha demostrado la existencia de una familia multigénica de BiP.

Se han llevado a cabo diversos estudios en los que la expresión de BiP de plantas responde a una variedad de estreses bióticos y abióticos como la sequía o la infección por hongos, ataque de insectos, estrés nutricional, temperatura y elicitores de la respuesta de patogénesis de la planta (Anderson *et al.*, 1994; Denecke *et al.*, 1995; Kalinsky *et al.*, 1995; Fontes *et al.*, 1996; Figueiredo *et al.*, 1997; Fontes *et al.*, 1999) (Fotografía 3). BiP está presente en condiciones normales de crecimiento, pero la transcripción de su gen se puede ver inducida por una variedad de estreses que conducen a la acumulación de proteínas anómalas en el RE. De hecho, la sobre-expresión de BiP en células cultivadas de mamíferos (Morris *et al.*, 1997) y protoplastos de tabaco (Leborgne-Castel *et al.*, 1999) inhibe la expresión de genes que son inducidos por la respuesta a las proteínas desnaturalizadas (UPR, del inglés, unfolded protein response) e incrementa la tolerancia al estrés, sugiriendo que BiP directamente mejora el estrés en el RE.

Fotografía 3. Niveles altos de BiP confieren tolerancia al estrés hídrico en plantas transgénicas 35S-BiPS7 de tabaco (generación T_1 resistente a la kanamicina) que fueron sometidas al mismo estrés que las plantas control mostradas en la parte inferior. (Alvim *et al*., 2001).

Por tanto, si nos basamos en la función de BiP como chaperona molecular y en la regulación de su expresión en respuesta a estreses, BiP puede actuar en ambos mecanismos. El papel protector de BiP frente al estrés hídrico se puede asociar con la protección de la estructura proteica y la integridad de la membrana, así como el mantenimiento de la elevada actividad secretora mediada por respuestas celulares originadas por el estrés hídrico (Ingram y Bartels, 1996). Además, BiP puede facilitar la formación y maduración de un grupo seleccionado de proteínas secretoras inducidas por estrés hídrico, que probablemente están implicadas en el mecanismo de respuesta osmótica (Alvim *et al*., 2001)

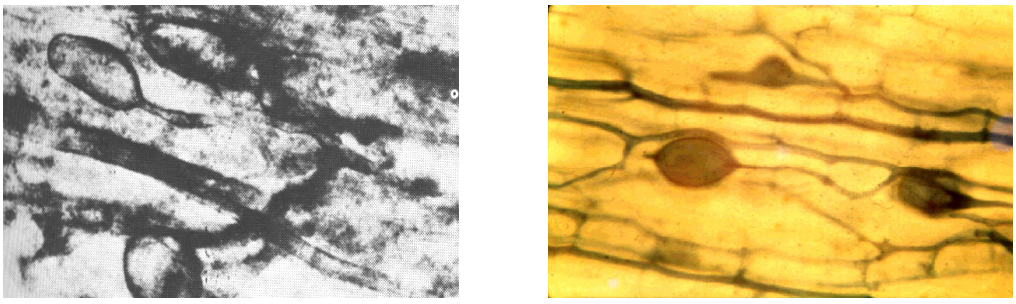
Desde el punto de vista de los hongos, la poca información existente se centra en levaduras. En *Saccharomyces cerevisiae*, BiP es codificado por el gen *KAR2*. La expresión del gen *KAR2* es inducida por estrés, choque térmico y tratamiento con tunicamicina, todos los cuales originan acumulación de proteínas mal ensambladas en el lumen del RE (Normintong *et al*., 1989). La proteína BiP KAR2 de *S. cerevisiae* es esencial para el ensamblaje de proteínas en el RE (Simons *et al*., 1995), para la translocación de precursores sintetizados *de novo* a través de la membrana del RE (Corsi y Schekman, 1997; McClellan *et al*., 1998) y para el transporte de regreso desde la membrana de polipéptidos aberrantes destinados a la degradación por el proteasoma (Plemper *et al*., 1997; Brodsky *et al*., 1999). Mutaciones en el gen *KAR2* originan una disminución del crecimiento a temperaturas elevadas y defectos en la translocación de proteínas al lumen del RE

3. LAS "MICORRIZAS"

3.1. Generalidades

El término "micorriza" (del griego "mikos", hongo, y "rriza", raíz) fue utilizado por vez primera por Frank a finales de siglo pasado (Frank, 1885) para hacer referencia a determinadas asociaciones existentes entre ciertos hongos del suelo y raíces de plantas. No obstante, fue en los años 50 cuando se comenzó a poner de manifiesto la importancia real y el significado de estas asociaciones, así como su presencia en la práctica totalidad de los sistemas suelo-planta (Bonfante-Fasolo, 1984; Barea y Jeffries, 1995). En la inmensa mayoría de los hábitats naturales (Barea y Azcón-Aguilar, 1983), incluso en las condiciones mas adversas (Mosse *et al.*, 1981; Hayman, 1982a), están micorrizadas aproximadamente un 97% de las plantas superiores (Harley y Smith, 1983). Es decir, sus raíces establecen asociaciones simbióticas mutualísticas con hongos específicos del suelo en las que ambos organismos se encuentran integrados de tal manera que dan lugar a una nueva entidad. Según esto, en la mayoría de los casos, el órgano de captación de nutrientes de la planta es la "micorriza", y no la "raíz" propiamente dicha (Harley y Smith, 1983).

Hoy se sabe que las micorrizas son tan antiguas como las propias plantas y que su origen se remonta al Ordivicio (hace 460 millones de años), como se deduce de la observación del primer registro fósil que se conoce (fósil Rhynie, datado en 370 millones de años) y la datación molecular (Redecker *et al.*, 2000).



Fotografía 4. Imagen que muestra el fósil Rhynie, de 370 millones de años (izquierda) e imagen mostrando las micorrizas arbusculares en la actualidad (derecha).

Atendiendo a las características morfológicas de la simbiosis, así como a los taxones a los que pertenecen las plantas y los hongos implicados, se pueden distinguir siete tipos de asociaciones micorrízica (Harley y Smith, 1983; Morton *et al.*, 1995). No obstante, las micorrizas arbusculares (MA), encuadradas dentro de las endomicorrizas o micorrizas endotróficas, llamadas así por colonizar intracelularmente la raíz, son las mas ampliamente distribuidas en la naturaleza. Aproximadamente el 90% de las especies vegetales terrestres las forman

(Kendrick y Berch, 1985; Trappe, 1986), las mayoría de ellas son de alto interés económico. El hecho de que la practica totalidad de las especies vegetales formen MA, contrasta con que sólo unas 150 especies de hongos las originan (Morton, 1988; Morton *et al.*, 1995), todos ellos pertenecientes al grupo de los Glomeromicetos (Schüβler *et al.*, 2001a). En estas asociaciones, la colonización de la raíz por las hifas del hongo es tanto inter- como intracelular, y se caracteriza porque en las células mas internas del córtex, las hifas del hongo, mediante divisiones dicotómicas sucesivas, dan lugar a unas formaciones caraterísticas denominadas arbúsculos, donde se produce el intercambio de nutrientes entre el hongo y la planta.

3.2. Las Micorrizas Arbusculares: Introducción

Las micorrizas arbusculares son el grupo más importante dentro de las simbiosis micorrícicas, tanto a nivel cuantitativo, como cualitativo, ya que entre las plantas que las forman se encuentran la práctica totalidad de las especies de interés agronómico e industrial (Hayman, 1982b; Linderman, 1988). Solo algunas familias, entre ellas las Chenopodiaceae, Cruciferae, Fumariaceae, Urticaceae y Poligonaceae, poseen especies que habitualmente no forman este tipo de simbiosis (Trappe, 1986).

En la actualidad, se ha acumulado una gran información acerca de esta simbiosis, especialmente en lo que se refiere a sus efectos sobre el crecimiento de la planta hospedadora. Los procesos fisiológicos y bioquímicos responsables de tales efectos son, en cambio, mucho menos conocidos. Hoy en día se sabe que las MA mejoran el enraizamiento y establecimiento de las plantas, facilitan el ciclado de nutrientes, protegen a las plantas frente a estreses bióticos y abióticos y mejoran la calidad del suelo, favoreciendo la diversidad y beneficiando la sucesión vegetal (Sánchez-Díaz y Honrubia, 1994; Ruiz-Lozano *et al.*, 1995a; Azcón-Aguilar y Barea, 1996). Por lo tanto, se considera que las MA desempeñan un papel crucial en la supervivencia y desarrollo de las plantas, sobre todo en suelos sometidos a condiciones de estrés (sequía, salinidad, cambios bruscos de temperatura, deficiencia de nutrientes), como los que caracterizan a los ecosistemas mediterráneos (Salamanca, 1991; Requena, 2001), así como en suelos degradados por procesos erosivos, incendios forestales, laboreo excesivo y contaminación.

3.3. Taxonomía de las micorrizas arbusculares

Los hongos implicados en la formación de las micorrizas arbusculares se consideraban hasta hace poco incluidos en el orden Glomales de los Zigomicetos, (Morton y Benny, 1990; Redecker *et al.*, 2000). Hoy día, en base al análisis de la subunidad pequeña del ARNr 18S se ha podido determinar su origen monofilético y se han agrupado en un nuevo phylum, denominado Glomeromycota. Este phylum se encontraría más próximo de los ascomicetos y basidiomicetos, con los que compartiría un ancestro común, que de los zigomicetos (Schüβler *et al.*, 2001a). Incluye una sola clase, los Glomeromycetes y cuatro órdenes: Glomerales, Diversisporales, Paraglomerales y Archaesporales como se refleja en la Figura 17B.

Un aspecto destacable de esta nueva clasificación es que el género *Glomus* se ha escindido en, al menos, tres grupos distintos: *Glomus* A, B y C. Los primeros de estos grupos están englobados en el orden Glomerales, mientras que *Glomus* C pertenece a los Diversisporales. Este hecho da idea de que bajo la aparente uniformidad de morfotipos de hongos micorrícicos se esconde una gran diversidad genética, y posiblemente, funcional aún por describir.

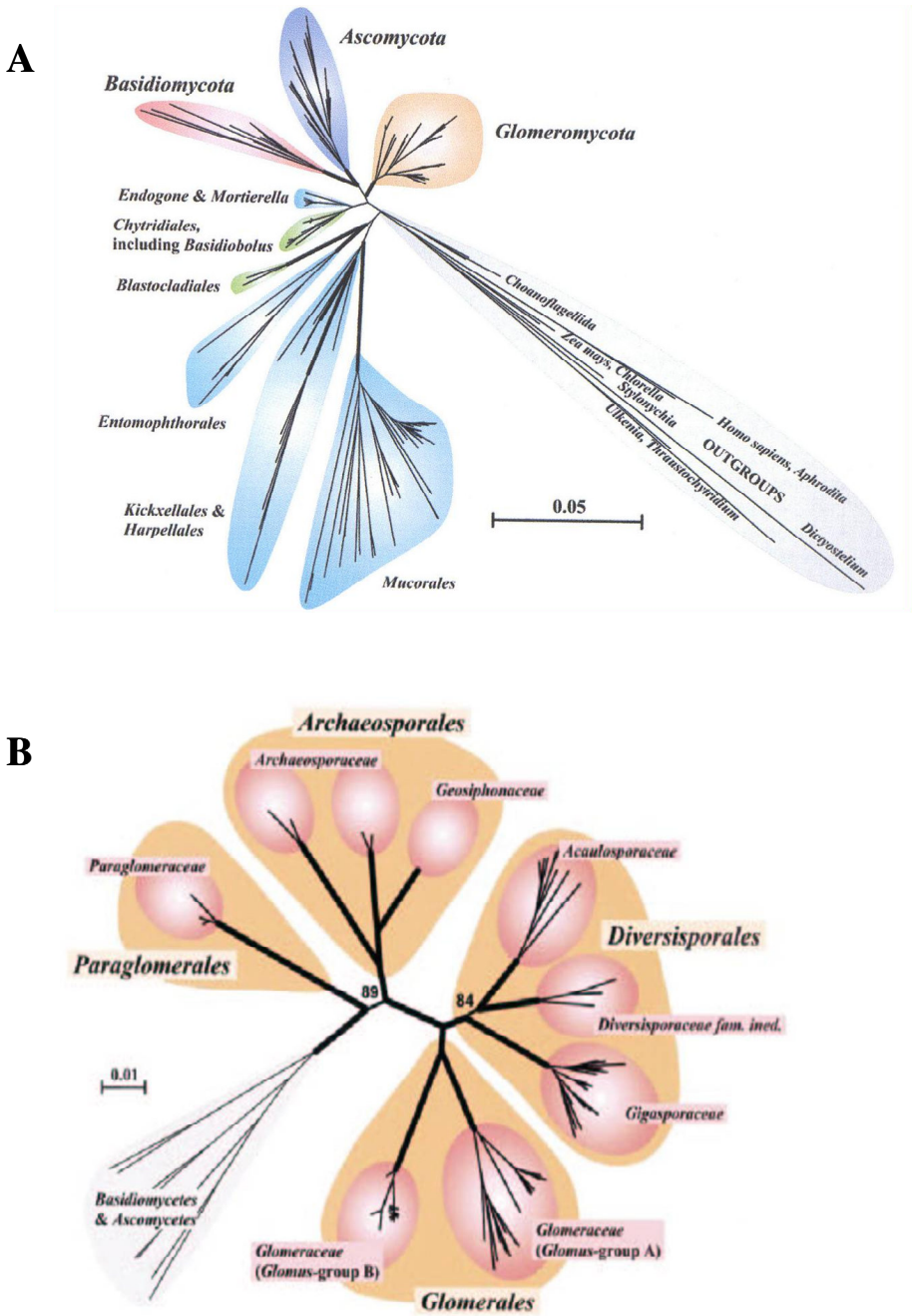


Figura 17. (A) Filogenia propuesta para los hongos MA basada en las secuencias de la subunidad pequeña (SSU) del ARNr. Tomado de Schüβler *et al*. (2001a). (B) Estructura taxonómica propuesta del phylum Glomeromycota en base a las secuencias de la SSU del ARNr.

La identificación de los hongos formadores de micorrizas arbusculares se ha basado hasta hace pocos años, en las características morfológicas y en el

desarrollo ontogénico de sus esporas multinucleadas (Gerdemann y Trappe, 1970; Morton, 1988; Morton y Benny, 1990; Walker, 1992). Sin embargo, cada vez hay más evidencias de que la identificación basada en criterios morfológicos tiene un uso limitado, ya que la baja diversidad morfológica de las esporas de los hongos micorrícicos no refleja la gran plasticidad fisiológica y la diversidad genética de sus poblaciones. El progreso que están experimentando en la actualidad las técnicas de biología molecular está posibilitando la utilización de criterios más constantes y fiables para caracterizar los hongos micorrícicos, facilitando así el subsiguiente análisis de la diversidad genética de las poblaciones (Helgason *et al.*, 1998; Schüβler *et al.*, 2001a, Clapp *et al.*, 2002). Debido a que el carácter de simbiontes obligados de estos hongos limita la cantidad de material fúngico disponible para ser analizado, la aplicación de técnicas basadas en la reacción en cadena de la polimerasa (en inglés, PCR) al estudios de los hongos micorrícicos ha supuesto un avance importnte en la caracterización genética de dichos microorganismos (Simon, 1996; Sanders *et al.*, 1996; Clapp *et al.*, 2002).

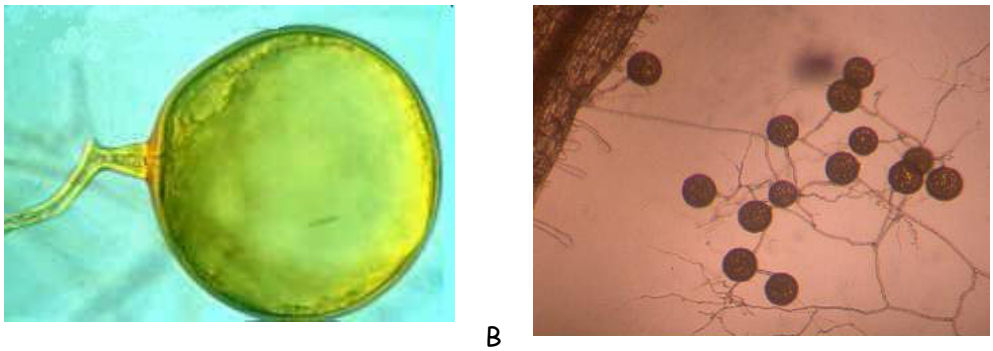
Técnicas como el análisis de los genes ribosómicos o metodologías innovadoras como PCR-SSCP (del inglés Polimerase Chain Reaction-Single Strand Conformational Polimorfism) o PCR-TGGE (del inglés Polimerase Chain Reaction-Temperature Gradient Gel Electrophoresis), están poniendo de manifiesto que las comunidades de hongos micorrícicos son mucho más diversas de lo que se pensaba (Chelius y Triplett, 1999; Daniell *et al.*, 2001; Fitter, 2001; Clapp *et al.*, 2002; Prosser, 2002). El estudio de la diversidad de las poblaciones de hongos micorrícicos es fundamental para saber cómo estos hongos afectan a la diversidad de las plantas y a su productividad (van der Heijden y Sanders, 2002), así como su contribución a la diversidad de funciones que llevan a cabo (Clapp *et al.*, 2002). Es obvio que los nuevos avances que se están produciendo en el análisis de la diversidad genética de los hongos formadores de micorrizas arbusculares están introduciendo un elevado grado de incertidumbre sobre el concepto de especie, e incluso de individuo, en estos hongos (Clapp *et al.*, 2002)

3.4. Formación de las micorrizas arbusculares

En el proceso de formación de la micorriza, durante el cual la planta acepta la colonización del hongo sin ejercer reacciones de defensa generalizadas (Dumas-Gaudot *et al.*, 2000), ocurren una serie de interacciones hongo-planta que van a dar lugar en último extremo a una integración morfológica y funcional de ambos simbiontes (Gianinazzi-Pearson *et al.*, 1996). Hoy se sabe que el establecimiento de la simbiosis es el resultado de un continuo diálogo molecular entre la planta y el hongo, ejercido por el intercambio de señales de reconocimiento (Vierheilig y Piché, 2002).

La identificación y clonación de los genes de la planta implicados, así como el conocimiento de los sistemas de señales que intervienen en la formación y funcionamiento de la simbiosis, son objeto de gran interés en la actualidad (Harrison *et al.*, 2000; Franken y Requena, 2001; Gollote *et al.*, 2002)

Se acepta que existen en el suelo tres formas de propágulos con diferente capacidad de supervivencia y potencial infectivo para establecer la simbiosis. Estas formas son las esporas, que constituyen las formas de resistencia de estos hongos, los fragmentos de raíces micorrizadas de plantas preexistentes y finalmente, las redes de hifas que sobreviven en el suelo. Las esporas de los hongos micorrícicos contienen numerosos núcleos y una gran cantidad de lípidos de reserva (Bécard y Pfeffer, 1993) (Fotografía 5) Se ha comprobado que las micorrizas, pueden mantener su capacidad infectiva después de permanecer en el suelo seco durante periodos de tiempo de hasta seis meses (Tommerup y Abbot, 1981).



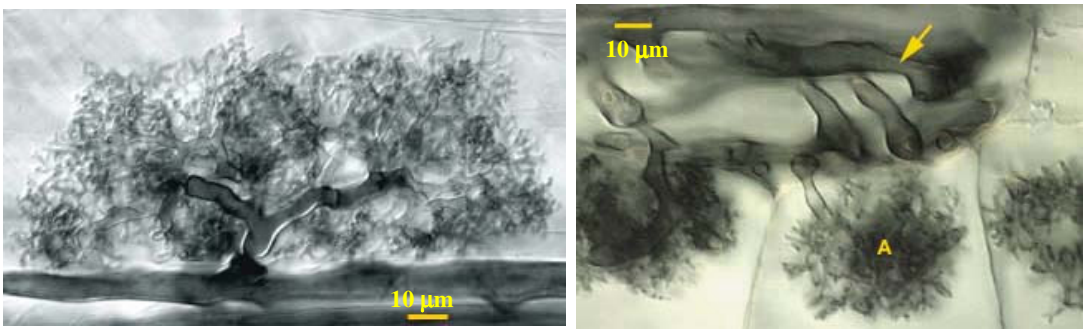
A B

Fotografía 5. (A) Espora de *Glomus mosseae*. Cortesía de Bundrett (B) Esporas de *Glomus intraradices*. Cortesía de P. Tiwari y A. Adholeya.

En condiciones favorables las **esporas germinan** y desarrollan el llamado tubo de germinación, que puede proliferar y formar un micelio que se extiende de forma radial y errática hasta alcanzar la rizosfera de una planta hospedadora susceptible de ser colonizada (Giovannetti, 2000; Giovannetti *et al.*, 2002). Las condiciones físicas del suelo, en especial humedad y temperatura, parecen ser los factores fundamentales que desencadenan la germinación de las esporas (Siqueira *et al.*, 1985). Las esporas de los hongos micorrícicos tienen almacenada toda la información genética y capacidad bioquímica y fisiológica para germinar, aunque determinadas condiciones físico-químicas, así como la presencia de exudados radicales y/o microorganismos del suelo pueden potenciar la germinación. Si las hifas "exploradoras" del hongo no encuentran las raíces de una planta hospedadora a las que colonizar, su citoplasma se retrae y la espora entra de nuevo en estado de quiescencia (Bago *et al.*, 1999) a la espera de encontrar una raíz que colonizar con éxito o bien degenere definitivamente. Una vez que una hifa "infectiva" alcanza la superficie de la raíz, se adhiere a ella y forma una estructura denominada "**apresorio**" a partir del cual se produce la penetración al interior de la raíz. Los mecanismos exactos por los cuales el hongo penetra son aún desconocidos. Se ha sugerido que la producción localizada de enzimas pectinolíticos y celulolíticos del hongo que degradan la pared celular de la planta lo justo para desorganizarla y no destruirla, junto al desarrollo de una presión física, podrían ser los mecanismos responsables de la entrada de las hifas en la raíz (García-Garrido *et al.*, 2000). En

general, se acepta que los sitios más habituales de penetración coinciden con los lugares más activos de la raíz, posiblemente porque la exudación radical es más abundante en estas zonas. El hongo micorrícico no penetra por heridas ni coloniza raíces muertas.

Una vez que la hifa colonizadora penetra en la raíz se ramifica intercelularmente constituyendo un micelio intrarradical y colonizando el córtex de la raíz de forma rápida, sin invadir endodermos ni meristemos. Simultáneamente tiene lugar una colonización intracelular, formándose las estructuras más características de la simbiosis, los "**arbúsculos**", mediante ramificación dicotómica repetida de hifas intracelulares. En cada célula sólo puede formarse un arbúsculo, que recibe este nombre porque su estructura recuerda a la de un pequeño árbol con tronco y ramificaciones (Fotografía 6).



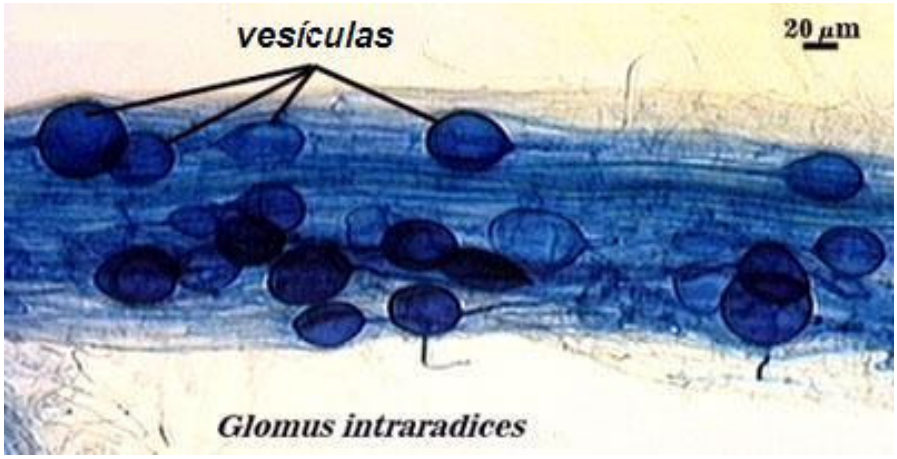
A B

Fotografía 6. (A) Imagen de la estructura de un arbúsculo de *Glomus mosseae* en *Allium porrum*. (B) Imagen de un arbúsculo –A- de *Glomus spp* en *Asarum canadense*.

Las hifas del hongo no penetran el plasmalema de la célula hospedadora sino que se produce una invaginación de éste que rodea las ramas del arbúsculo. La interfase entre los dos simbiontes queda en esencia constituida por el plasmalema de la célula hospedadora o membrana peri-arbuscular, una matriz interfacial, la pared celular del hongo y su membrana plasmática (Bonfante-Fasolo *et al.*, 1986; Balestrini *et al.*, 1996; Gianinazzi-Pearson *et al.*, 1996; Bonfante, 2001) la barrera que suponen las paredes entre ambos simbiontes queda por lo tanto reducida a un mínimo (Gianinazzi-Pearson *et al.*, 1991). El arbúsculo representa una extensa superficie de contacto entre ambos simbiontes. Se asume que el papel fundamental de esta interfase es el de facilitar el intercambio bidireccional de nutrientes entre el hongo y la planta (Smith *et al.*, 1994). En la célula vegetal colonizada tiene lugar un aumento del contenido citoplasmático, el núcleo aumenta de volumen, migra al centro de la célula y aumenta la actividad transcripcional (Smith y Gianinazzi-Pearson, 1988). La vida media del arbúsculo es de 7 a 14 días y una vez que degenera, la célula vegetal recupera la situación previa a la colonización, e incluso puede ser colonizada de nuevo. El colapso arbuscular podría estar inducido por el

reconocimiento por parte de la planta del arbúsculo como una estructura extraña (García-Garrido y Ocampo, 2002).

Algunas especies de hongos pueden formar también unas estructuras globosas llamadas **vesículas** con un alto contenido en lípidos y que parecen constituir un órgano de reserva de nutrientes (Barea *et al.*, 1991) (Fotografía 7). Las vesículas no son efímeras como los arbúsculos, sino que desde que aparecen van madurando y parece ser que, en algunas ocasiones, pueden llegar a convertirse en esporas del hongo. La transformación de estas vesículas en esporas podría estar ligada a situaciones de estrés para la micorriza, o a la muerte inminente de la planta.



Fotografía 7. Vesículas de *Glomus intraradices* a lo largo de la raíz colonizada teñida con tripan blue. INVAM.

El desarrollo del hongo en el interior de la raíz va acompañado de una proliferación de hifas en el suelo que la rodea formando lo que se denomina el micelio externo o extrarradical. Este micelio funciona como un sistema radical complementario, fundamental para la adquisición de nutrientes y agua por la planta (Barea, 2000). Se estima que por cada cm de raíz micorrizada se puede producir hasta 1 m de hifas. Su función es explorar microhábitats del suelo inaccesibles para las raíces. Sobre las hifas extrarradicales se pueden formar esporas de resistencia, con lo cual se cierra el ciclo de vida del hongo. Las hifas extrarradicales del hongo pueden además formar nuevos puntos de entrada sobre la superficie de la raíz y contribuir así a la generalización de la colonización micorrícica.

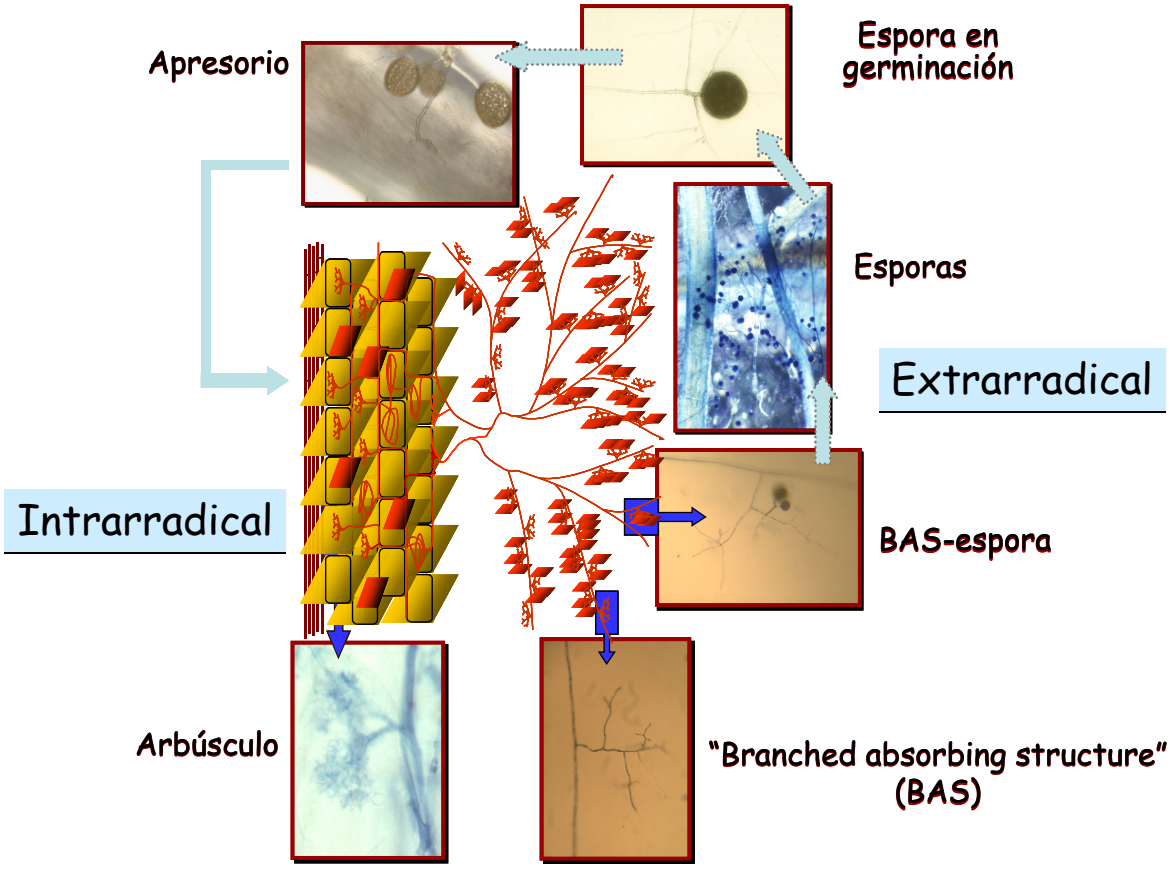


Figura 18. Ciclo de vida de *Glomus intraradices*.

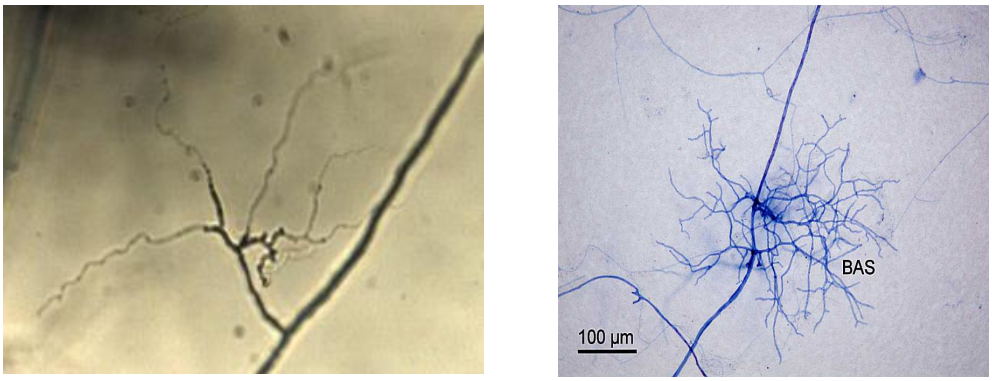
3.5. Morfología de la simbiosis MA

La formación de los arbúsculos supone una alteración profunda de la célula vegetal, lo que se manifiesta principalmente por la deformación y proliferación del plasmalema (Alexander *et al.*, 1988) para acomodar al arbúsculo, el cual nunca penetra al citoplasma. Se produce igualmente una reordenación del citoesqueleto (Genre y Bonfante 1998; Matsubara *et al.*, 1999), lo que conlleva un cambio en la posición del núcleo, que pasa a una posición central, y una fragmentación de la vauola (Bonfante y Perotto, 1995). Se observa, asimismo una menor heterocromaticidad del núcleo de la célula, lo que indica una mayor actividad transcripcional y un aumento del metabolismo (Balestrini *et al.*, 1994).

No sólo la célula vegetal se altera, el propio hongo experimenta cambios en su estructura que se manifiestan en una progresiva reducción del grosor de la pared celular a medida que se producen las ramificaciones más finas, pasando de unos 500 nm de grosor en las hifas intercelulares, a los 50 nm en los ápices de los arbúsculos (Bonfante -Fasolo *et al.*, 1992). La observación de que en la membrana periarbuscular se localizan transportadores de fosfato específicos de la simbiosis (Rausch *et al.*, 2001) y una H^+-ATPasa (Gianinazzi-Pearson *et al.*, 2000), indica que

es a nivel de los arbúsculos donde se produce el intercambio de nutrientes entre la planta y el hongo.

Se ha podido observar que durante el desarrollo del micelio externo, las hifas exploradoras que lo constituyen y que son las responsables del avance del micelio y de la extensión de la colonia fúngica, forman a intervalos regulares estructuras muy ramificadas. Estas estructuras son conocidas hoy día como BAS (del inglés Branched Absorbing Structures, Estructuras ramificadas de absorción), cuya hipotética función sería la absorción de nutrientes del suelo (Bago 1998, 2000) (Fotografía 8).



Fotografía 8. BAS dispuestos a lo largo de las hifas del micelio del hongo MA.

Cabe destacar que, en contraste con el micelio intrarradical, que se encuentra protegido por los tejidos de la raíz de la planta, el micelio extrarradical, al desarrollarse directamente en el suelo, se encuentra mucho más expuesto a las condiciones ambientales, y a la acción de otros microorganismos del suelo. Con ellos va a desarrollar una serie de interacciones de gran importancia para el desarrollo de las plantas, el equilibrio de las poblaciones microbianas y la formación de agregados estables en el suelo y el mantenimiento de su estructura (Jeffries y Barea, 2001).

3.6. Fisiología de las micorrizas arbusculares

El carácter mutualista de la simbiosis supone que ambos simbiontes resultan beneficiados de esta asociación, lo que generalmente se traduce en una mejor nutrición de ambos. A continuación se resumen los aspectos fundamentales del funcionamiento de la simbiosis, referidos principalmente al intercambio de nutrientes entre ambos simbiontes, y su papel en la absorción y movilización de micronutrientes.

Metabolismo del carbono en los hongos micorrícicos

La primera evidencia experimental sobre la transferencia de compuestos carbonados de la planta al hongo fue proporcionada por Ho y Trappe (1973), quienes usando $^{14}CO_2$ demostraron que, tras unas semanas, se detectaba C marcado en el micelio del hongo. Actualmente se sabe que el micelio intrarradical es capaz de captar fructosa y glucosa (Shachar-Hill *et al.*, 1995; Pfeffer *et al.*, 1999), que son rápidamente transformadas en trehalosa y glucógeno, con el fin de disminuir la osmolaridad. Parte de estos carbohidratos son transformados en el micelio intrarradical en compuestos lipídicos, que posteriormente serán transportados al micelio extrarradical (Bago *et al.*, 2002), donde mediante gluconeogénesis se transformarán en carbohidratos. El micelio extrarradical depende para su desarrollo de este aporte de productos carbonados dada su incapacidad para absorber hexosas del medio (Bago *et al.*, 2000). En los hongos micorrícicos se produce una compartimentación del metabolismo, de forma que las capacidades de síntesis lipídica sólo residen en el micelio intrarradical, mientras que las capacidades gluconeogénicas se localizan únicamente en el micelio extrarradical. Esta marcada compartimentación del metabolismo del hongo, asociada a la diferenciación que experimenta el micelio al establecer la simbiosis, podría estar en la base de la incapacidad del hongo para crecer independientemente de la planta.

Nutrición mineral de las micorrizas

Fue a mediados del siglo pasado cuando empezó a cobrar interés el estudio de las causas del mejor crecimiento de las plantas micorrizadas. El primer trabajo sobre el tema señalaba cómo el manzano micorrizado presentaba un mayor contenido en Fe y Cu que el no micorrizado cuando crecía en suelos deficientes en estos micronutrientes (Mosse, 1957). Posteriormente se puso de manifiesto que los hongos formadores de micorrizas arbusculares mejoran también la absorción de fosfato por la planta (Gerdermann, 1964; Daft y Nicolson, 1966; Baylis, 1967) y que son capaces de transferir N a la planta mediante absorción de NH_4^+ (Johansen *et al.*, 1992; Frey y Schüepp, 1993), o NO_3^- (George *et al.*, 1992; Tobar *et al.*, 1994a; Bago *et al.*, 1996) del suelo circundante, y su posterior transferencia.

(a) Nutrición fosforada

La absorción del fosfato es un mecanismo altamente eficiente, lo que se pone de manifiesto por la rápida incorporación de fósforo por las hifas del micelio extrarradical, requiriéndose tan sólo tres horas para alcanzar los niveles máximos de acumulación de polifosfato (Ezawa *et al.*, 2004) (Figura 19). Esta capacidad de incorporación es fruto de la existencia de transportadores de alta afinidad de fosfato en el micelio extrarradical (Harrison y Van Buuren, 1995).

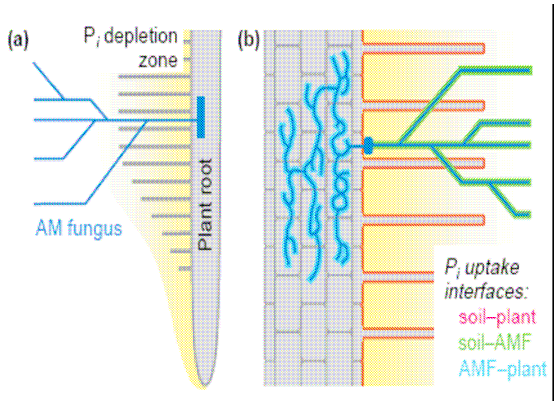


Figura 19. Transporte del P en plantas micorrizadas. (a) La raíz de la planta crea una zona de agotamiento de P causada por la captación de P y la baja tasa de difusión de P en el suelo. El micelio extrarradical del hongo MA crece hacia la zona de agotamiento, alcanzando un nuevo pool de P soluble. (b) La interfase implica la captación de P en la simbiosis MA. Las interfase suelo-planta, suelo-hongoMA y hongo MA-planta se puestran en rojo, verde y azul respectivamente. La translocación transmembrana del P está mediada por transportadores de P que residen el la membrana de las células de la correspondiente interfase. (Adaptado de Karandashov y Bucher, 2005).

Con el fin de evitar la acumulación de iones fosfato, que dificultarían el funcionamiento normal del hongo mediante un incremento de la presión osmótica, estos se polimerizan formando cadenas de polifosfatos de unas 17 unidades aproximadamente, que se acumulan sobre todo en las vacuolas (Rasmussen *et al.*, 2000). El fosfato es transportado como polifosfato al micelio intrarradical y una vez allí, es hidrolizado, liberándose el fosfato mediante la actividad de ciertas fosfatasas alcalinas presentes en la vacuola (Gianinazzi-Pearson y Gianinazzi, 1978).

Aún no se conoce el mecanismo de extrusión del fosfato del micelio intrarradical a la matriz interfacial de la interfase simbiótica que se desarrolla en las células corticales colonizadas por los arbúsculos, sin embargo, diversos estudios (Rausch *et al.*, 2001; Harrison *et al.*, 2002) indican que la transferencia de fosfato ocurre preferentemente a nivel de dichas células.

(b) Nutrición nitrogenada

Además de transferir nitrógeno a la planta como NH_4^+ o NO_3^-, hay indicios de cierta capacidad de transporte de N orgánico (Hodge *et al.*, 2001), especialmente aminoácidos (Hawkins *et al.*, 2000).

Los hongos micorrícicos prefieren como fuente de N el amonio frente al nitrato (Johansen *et al.*, 1992). La incorporación de amonio se lleva a cabo mediante transportadores específicos. El amonio absorbido sería rápidamente incorporado a glutamato, para dar glutamina, mediante distintas vías, aunque parece más probable el ciclo de la Glutamina sintasa/Glutamato sintasa , ya que

estas actividades se han detectado en hongos micorrícicos (Johansen *et al.*, 1996; Breuninger *et al.*, 2004).

La asimilación del nitrato parece estar facilitada por la enzima nitrato reductasa, cuya actividad se ha detectado en esporas (Ho y Trappe, 1975) y en extractos de raíces micorrizadas (Subramanian y Charest, 1998). Igualmente se ha demostrado que los hongos MA poseen el gen que codifica la nitrato reductasa (Kaldorf *et al.*, 1994) y que dicha actividad se localiza en los arbúsculos (Kaldorf *et al.*, 1998). Esta actividad nitrato reductasa permitiría la reducción del nitrato y su posterior incorporación a la glutamina en forma de amonio.

La transferencia del N a la planta ocurre en un proceso asociado al ciclo de la urea y al transporte de polifosfato, propuesto por primera vez por Bago *et al.* (2001) y recientemente confirmado por Govindarajulu *et al.* (2005).

3.7. Significado de las micorrizas en el sistema suelo-planta

Las micorrizas llevan a cabo diversas actividades relevantes desde el punto de vista de la sostenibilidad, ya que pueden representar una reducción en el aporte de fertilizantes y fitofármacos, y contribuir así a la conservación o establecimiento de sistemas sostenibles ya sean agrícolas, forestales o paisajísticos (Jeffries y Barea, 2001). Las micorrizas realizan las siguientes acciones en los sistemas suelo-planta:

- Incrementan el aporte de nutrientes a las plantas

El efecto beneficioso más y mejor estudiado que ejercen las micorrizas sobre las plantas es, sin duda, el que conduce a una mejora del crecimiento así como de su estado nutricional.

Mediante la utilización de isótopos y sistemas compartimentados, en los que la hifas se desarrollan en compartimentos a los que las raíces no tienen acceso, se ha confirmado que el micelio externo de los hongos micorrícicos puede absorber P, Zn, NO_3^- y NH_4^+ de microhábitats distantes hasta 25 cm de la superficie de la raíz y transferírselos a las plantas con las que se asocian (Pearson y Jackobsen, 1993; Bürker y Robson, 1994; Tobar *et al.*, 1994a; Smith *et al.*, 2000). Los efectos directos de la simbiosis sobre la nutrición mineral de las plantas se manifiestan principalmente para aquellos nutrientes que son poco móviles y que están presentes en bajas concentraciones en la solución del suelo. El ejemplo característico es el P. Debido a la mayor absorción y al uso más eficiente de nutrientes en plantas micorrizadas, estas presentan generalmente una mayor biomasa que sus respectivas plantas control. Además, y debido a esa mayor eficiencia en la absorción de nutrientes, la relación parte aérea/raíz es mayor en plantas micorrizadas.

- Protegen a la planta frente a estreses abióticos

La simbiosis micorrícica contribuye a incrementar la resistencia/tolerancia de las plantas a la sequía, salinidad, estados de deficiencia de nutrientes, exceso de metales pesados, degradación del suelo, etc.

- Incrementan la actividad fotosintética de la planta

La colonización micorrícica estimula normalmente la actividad fotosintética de las plantas. Así lo avalan numerosos trabajos de investigación (Wright *et al.*, 1998; Valentine *et al.*, 2001).

- Mejoran el enraizamiento de las plantas

Esta actividad está basada en la producción de hormonas, vitaminas u otras sustancias fitoactivas por parte de los hongos.

- Mejoran la estructura del suelo

Las hifas de los hongos micorrícicos están implicadas en la formación de agregados estables del suelo, un aspecto clave de la calidad del mismo. El micelio extrarradical y las propias raíces actúan como nexo de unión de las partículas orgánicas e inorgánicas del suelo, mientras que los polisacáridos, de origen fundamentalmente bacteriano, actúan como aglomerantes de las estructuras formadas (Tisdall *et al.*, 1997; Miller y Jastrow, 2000).

- Favorecen la diversidad de las comunidades de plantas y la sucesión vegetal

Cada planta muestra un nivel de compatibilidad mayor con determinados ecotipos de hongos micorrícicos, por lo que la conservación de la diversidad de estos hongos, beneficia la diversidad y sucesión de las plantas.

- Control integrado de patógenos

En los últimos tiempos despierta un elevado interés el papel que las micorrizas ejercen confiriendo una mayor tolerancia/resistencia a las plantas frente al ataque de patógenos que causan enfermedades a los cultivos. Tales efectos son difíciles de generalizar y dependen en gran medida de la especie vegetal implicada, del hongo MA, el patógeno y su nivel de virulencia y de las condiciones medioambientales (Hooker *et al.*, 1994; Azcón-Aguilar y Barea, 1996). En general se ha descrito que las micorrizas reducen los síntomas cuando se trata de enfermedades que afectan al sistema radical (Cordier *et al.*, 1996; Slezack *et al.*, 2000). Una condición imprescindible para que se manifieste esta protección es que la simbiosis esté establecida antes de que se produzca el ataque del patógeno (Azcón-Aguilar y Barea, 1996).

4. SIMBIOSIS MICORRICICO ARBUSCULAR Y TOLERANCIA A ESTRÉS HÍDRICO

Probablemente los efectos de las micorrizas arbusculares no sean tan determinantes sobre las relaciones hídricas de las plantas como lo son sobre la adquisición de P y el crecimiento de la planta hospedadora. Sin embargo, si esos efectos se mantienen, pueden tener un efecto significativo sobre el funcionamiento de la planta (Augé 2001) en condiciones de déficit hídrico.

La tolerancia a la sequía de las plantas micorrizadas puede ser consecuencia de la captación de agua por el micelio externo (efecto directo) y/o de la mejora del estatus nutricional de la planta hospedadora (efecto indirecto), especialmente fósforo (P) y nitrógeno (N) (Fitter 1988, Subramanian y Charest 1999). Además, las plantas micorrizadas se recuperan del estrés hídrico más rápidamente y tienen mayor capacidad para extraer agua del suelo incluso con un bajo potencial hídrico (Subramanian *et al*. 1997). Otros estudios sugieren que el uso del agua en la planta hospedadora está regulado por cambios en las fitohormonas (Duan *et al*. 1996) o en la morfología de la raíz (Kothari *et al*. 1990). La tolerancia a la sequía en la planta micorrizada es un fenómeno complejo que engloba factores de la planta (bioquímicos, nutricionales y morfológicos) y factores del suelo (estructural, nutricional y biológico). En cualquier caso, en la actualidad se acepta que la mejora de la tolerancia de las plantas micorrizadas frente al déficit hídrico se debe a la combinación de efectos físicos, nutricionales, fisiológicos y celulares (Ruiz-Lozano, 2003)

Los estudios realizados en los últimos años han permitido sugerir posibles mecanismos a través de los cuales la simbiosis MA incrementa la tolerancia de las plantas frente al déficit hídrico. Entre estos mecanismos destacan los que vamos a tratar a continuación.

Fotografía 9. Plantas de tomate no MA (izquierda) y MA (derecha)

4.1. Absorción y transferencia de agua a través de las hifas del hongo

El micelio extrarradical (MER) se despliega por la rizosfera y transporta agua que difícilmente está disponible para las raíces. Las hifas del hongo MA, con un diámetro de 2-5 μm, pueden penetrar en poros del suelo que resultan inaccesibles incluso para las raíces más finas (10-20 μm de diámetro). Los estudios pioneros de Allen (1982) sugiriendo la posible intervención del micelio extrarradical en la captación y translocación de agua directamente a las raíces, fueron confirmados con posterioridad en experimentos en los que se cultivaron plantas en sistemas compartimentalizados y aplicando agua en un compartimento donde sólo las hifas fúngicas tenían acceso (Ruiz-Lozano y Azcón, 1995). Kothari *et al.* (1990) mostró grandes diferencias en la captación de agua entre raíces de maíz MA (1,3 ml agua cm^{-1} raíz s^{-1} x 10^{-7}) y no MA (0,61 ml agua cm^{-1} raíz s^{-1} x 10^{-7}). Hardie y Leyton (1981) observaron que la conductividad hidráulica en raíces de trébol fue de 2 a 3 veces mayor en plantas MA que en plantas no MA, lo que sugiere que el hongo MA transporta una cantidad de agua considerable.

En un trabajo de Marulanda *et al.* (2003) se demostró que existían diferencias entre los distintos hongos MA en la capacidad de captar y transferir agua a la planta hospedadora. Los más efectivos fueron especies como *Glomus intraradices* , *Glomus coronatum* o *Glomus claroideum*, mientras que especies como *Glomus constrictum* o *Glomus geosporum* resultaron mucho menos efectivas. La mayor capacidad de los primeros para captar y transferir agua a la planta hospedadora se relacionó con la mayor producción de micelio extrarradical de estos hongos, lo que permitió una mejor exploración y explotación de los recursos hídricos disponibles.

En este contexto, es interesante destacar que estudios más recientes han demostrado la inducción de la expresión de genes que codifican proteínas MIP o aquaporinas en la simbiosis MA (Roussel *et al.*, 1997, Krajinski *et al*, 2000). No obstante, estos últimos estudios se han realizado en condiciones de adecuada disponibilidad hídrica para la planta, y la conexión de estas aquaporinas inducidas por la simbiosis MA con la capacidad de los hongos para captar y transferir agua a la planta hospedadora en condiciones de sequía no ha sido aún analizada. Dado que se ha propuesto que las aquaporinas intervienen en la osmorregulación celular (Kjelbom *et al.* 1999), habría que estudiar si dicha sobre expresión tiene algún efecto incrementando la permeabilidad al agua en la simbiosis MA o si, como es de esperar, juega algún papel en las relaciones hídricas de las plantas micorrizadas en condiciones de sequía.

4.2. Mejora del ajuste osmótico

A medida que un suelo se seca y el potencial hídrico del mismo va tomando valores cada vez más negativos, las plantas han de disminuir su potencial hídrico a fin de mantener el gradiente de potencial necesario para la absorción de agua. El mecanismo más importante para conseguir este efecto es la disminución del potencial osmótico mediante la acumulación activa de iones inorgánicos o de solutos orgánicos, fenómeno conocido como osmorregulación (Morgan 1984; Sánchez-Díaz y Aguirreolea, 1993; Hoekstra *et al.*, 2001). El ajuste osmótico posibilita así el

mantenimiento, en condiciones de sequía, de la turgencia y de los procesos dependientes de la misma, tales como expansión y crecimiento celular, apertura estomática, fotosíntesis, etc.

Por lo general, los solutos que participan en el ajuste osmótico son iones inorgánicos (especialmente K^+ y Cl^-) y solutos orgánicos sin carga que se acumulan en el citoplasma, entre los que destacan la prolina y la betaina, aunque se pueden acumular también azúcares reductores, sacarosa, pinitol o manitol (Antolín y Sánchez-Díaz, 1992).

Los estudios realizados hasta la fecha en relación con la acumulación de sustancias osmorreguladoras en la simbiosis MA son escasos, si bien se ha observado un incremento en la acumulación de prolina en las plantas micorrizadas en condiciones de sequía respecto a los controles sin micorrizar (Ruiz-Lozano *et al.*, 1995; Ruiz-Lozano y Azcón, 1997; Goicoechea *et al.*, 1998). Estos estudios también han demostrado que el incremento en la acumulación de prolina es bastante variable dependiendo del hongo MA implicado. Así por ejemplo, mientras las plantas colonizadas con *G. deserticola* acumularon 120 nmol de prolina por gramo de peso fresco, las plantas colonizadas por *G. intraradices* acumularon solo 41 nmol (Ruiz-Lozano *et al.*, 1995)

El estrés hídrico modifica el metabolismo de la planta y puede afectar al contenido de azúcares, proteínas solubles y aminoácidos. Se ha observado que los **azúcares solubles** de plantas MA se incrementan bajo condiciones de déficit hídrico (Porcel y Ruiz-Lozano 2004). La proporción y el alcance del incremento de azúcares dependen de las condiciones ambientales. La acumulación de azúcares es extensamente considerada como una respuesta adaptativa a la sequía (Munns 1988). La MA parece incrementar el almidón (Augé *et al.*, 1987b, Schellenbaum *et al.*, 1998) o azúcares (Porcel *et al.*, 2003) bajo condiciones de sequía. La acumulación de azúcares solubles puede colaborar en el ajuste osmótico y contribuir a la tolerancia de la planta hospedadora (Porcel *et al.*, 2003) de manera que los azúcares totales en plantas de soja MA sometidas a estrés son un 20% más que en raíces no MA, lo que contribuye a mantener el turgor mediante el ajuste osmótico. Igualmente, las **proteínas solubles** en plantas sometidas a estrés tienen un papel vital y contrarrestan la entrada de N reducido por procesos vegetativos o reproductivos (Hanson y Hitz, 1982). La inhibición de la síntesis de proteínas en plantas es una respuesta temprana al estrés hídrico (Hsiao, 1973). Pelah *et al.* (1997) mostraron que la tolerancia a la sequía de especies resistentes, *Populus popularis*, comparada con una menos tolerante, *P. tomentosa*, era positivamente correlacionada con la acumulación de un set de proteínas inducidas por el déficit hídrico. Durante la simbiosis MA, se han detectado nuevas proteínas llamadas micorricinas de origen fúngico y/o vegetal (Hilbert *et al.*, 1991). Estas micorricinas (16-78 KDa) se han detectado en soja (Pacovsky, 1989), cebolla (Dumas *et al.*, 1990), tomate (Simoneau *et al.*, 1994), trébol rojo (Arines *et al.*, 1993) y raíces transformadas de zanahoria (Toussaint *et al.*, 2004), aunque actualmente sus funciones no están del todo establecidas. Tras la sequía, se detectó un incremento de la concentración de proteínas en parte aérea de plantas

MA (Ruiz-Lozano y Azcón, 1996; Subramanian y Charest, 1999), y también en condiciones de estrés por frío (Charest *et al.*, 1993; Paradis *et al.*, 1995).

4.3. Cambios en las propiedades del suelo y en la capacidad de retención de agua

Los hongos pueden ser los organismos del suelo más efectivos en la estabilización de estructuras del suelo (McCalla, 1946; Swaby, 1949; Foster, 1994), y a menudo, los hongos MA ocupan la porción más grande de la biomasa microbiana del suelo (Hayman, 1978). Las hifas fúngicas de la MA crecen por la matriz del suelo y crean un esqueleto que alberga partículas del suelo. El hongo de la micorriza es un agregador efectivo y su funcionamiento se considera un factor biológico en la mejora de la estructura y arquitectura del suelo. Las hifas externas alteran las propiedades físicas y químicas del suelo (Smith y Read, 1997). Estos asociados de hifas producen con frecuencia exudados que originan partículas del suelo que se adhieren y forman agregados (Oades, 1993; Reichenbach y Schonbeck, 1995). El entramado físico del MER es uno de los principales mecanismos de agregación. La estructura del suelo dirige el movimiento de agua y gases en el suelo, que afecta al patrón de humedad reducido del suelo. Subramanian et al. (1997) mostraron que el suelo con MA retenía una mayor humedad durante 3 semanas sometido a ciclos de sequía. Reichenbach y Schonbeck (1995) observaron que el crecimiento intensivo de las hifas en la zona de la raíz de ciertas plantas MA, mejora el volumen de los poros del sustrato, el cual incrementa la capacidad de retener agua en el suelo. Así, la colonización MA, mediante la mejora de las condiciones físicas y químicas del suelo favorece la tolerancia a la sequía en las plantas. Además de las condiciones físicas del suelo, la colonización micorrícica incrementa la disponibilidad de nutrientes. La colonización MA incrementa el contenido de materia orgánica del suelo en un corto período de tiempo (Quintero-Ramos *et al.*, 1993). Cheshire *et al.*, (1984) encontró que los efectos de agregación de la materia orgánica estaban asociados con su contenido en polisacáridos. La disponibilidad de N y P en suelo aumentó sustancialmente en presencia del hongo MA independientemente de las deficiencias de nutrientes o de la intensidad de la sequía (Subramanian y Santhanakrishnan, 2003). La micorriza también afecta a la rizosfera alterando otras comunidades microbianas. Ya que las plantas MA son nutricionalmente ricas, estas plantas pueden modificar la calidad y cantidad de los exudados radicales, originando un nuevo equilibrio microbiano en el suelo de la rizosfera. Los efectos físicos y químicos del hongo llegan al suelo para crear una nueva dimensión, tanto espacial como biológica (Linderman, 1992). El MER exuda materia orgánica usada como sustrato para otros microorganismos del suelo. Secilia y Bagyaraj (1987) detectaron mayor población de bacterias en suelo inoculado con micorriza que en el no micorrizado acompañado de una alteración de las relaciones hídricas de la planta en condiciones de sequía.

Augé et al., (2001) investigaron si la simbiosis MA influía sobre las propiedades de retención de la humedad del suelo y vieron que 7 meses de micorrización por *G. intraradices* alteraba las características de la curva relativa

de humedad del suelo en comparación con suelo "no micorrícico" con una densidad radical similar. Dentro del rango de –1 a –5 MPa, el suelo "micorrícico" tuvo que secarse más que el suelo "no micorrícico" para conseguir el mismo potencial mátrico del suelo. El estudio reveló que el suelo "micorrícico" tuvo más agregados estables y una densidad de hifas extrarradicales mayor que el suelo "no micorrícico", lo que se correlacionó con unas propiedades de retención de la humedad del suelo "micorrícico" mejoradas. Augé et al. (2001; 2004) propusieron que los efectos observados en el intercambio gaseoso de las plantas micorrizadas podrían deberse a una influencia de la simbiosis MA sobre el suelo. Las acciones físicas, químicas y biológicas de las hifas MA y de sus exudados afectan a la estructura del suelo, lo cual afecta, a su vez, a las propiedades de retención de agua del mismo. Por lo tanto, propusieron que la simbiosis MA podía tener un efecto sobre la capacidad de retención de agua de los suelos en los que crecen plantas micorrizadas. Los resultados obtenidos en sus estudios demuestran que la simbiosis MA incrementa la formación de agregados estables de suelo y la densidad de hifas fúngicas en el mismo, lo cual se traduce en un incremento significativo de la capacidad de retener agua de los suelos donde crecen plantas micorrizadas frente a suelos desprovistos de micorrizas.

4.4. Mejora del intercambio gaseoso y de las relaciones hídricas de la planta hospedadora

Un gran número de estudios ha demostrado que durante la desecación del suelo, las plantas micorrizadas a menudo mantienen intercambios gaseosos más elevados que las plantas no micorrizadas de similar porte y estatus nutricional (Augé *et al.*, 1987a; Bethlenfalvay *et al.*, 1988; Augé *et al.*, 1992; Sánchez Díaz y Honrubia 1994; Ruiz-Lozano *et al.*, 1995a, 1995b; Goicoechea *et al.*, 1997). Las plantas micorrizadas y no micorrizadas frecuentemente muestran distintos puntos críticos en los umbrales de conducta de los estomas durante períodos de sequía. Por ejemplo, el potencial hídrico de la hoja en el momento de iniciarse el cierre estomático fue 0.2 MPa más bajo en plantas de trigo colonizadas por *Glomus fasciculatum* que en plantas no micorrizadas de tamaño similar (Allen y Boosalis, 1983). De forma similar, el potencial hídrico de la hoja en el momento del cierre de los estomas fue 0.7 MPa más bajo en plantas de rosa colonizadas por *G. deserticola* o *G. intraradices* que en plantas no micorrizadas de tamaño similar (Augé *et al.*, 1986). Duan *et al.* (1996) encontraron que las plantas micorrizadas mantienen más elevada la conductancia estomática, rango de transpiración y agua en la parte aérea que las plantas no micorrizadas. El estatus hídrico foliar más alto se asoció con unas concentraciones más bajas de ácido abscísico (ABA) en la savia del xilema y unos flujos de ABA más bajos en las hojas de plantas micorrizadas bajo condiciones hídricas limitantes. El mecanismo por el cual la simbiosis MA consigue este efecto no está claro, pero Duan et al. (1996) han sugerido que el hongo MA probablemente incrementa la capacidad del sistema radical del captar agua del suelo seco, originando una conductancia estomática más alta y un contenido hídrico mayor en parte aérea. Por otra parte, las evidencias proceden de otros estudios

donde se ha visto que las micorrizas arbusculares pueden influir directamente en la transpiración de las hojas, incluso después de que éstas hayan sido separadas de la planta (Green *et al.*, 1998)

La explicación más obvia para los cambios en el balance hídrico de la planta y la resistencia a la sequía inducidos por la MA suelen ir asociados con el tamaño de la planta y la fenología de esta, mediada por el incremento en la adquisición de P y a veces, de otros nutrientes. El tamaño de la planta puede afectar a sus relaciones hídricas, y la simbiosis MA a menudo afecta al tamaño de la planta, de modo que plantas más grandes con sistema radical más desarrollado pueden tener acceso a reservas de agua más lejanas (Fitter 1985; Koide 1993).

La hidratación de la planta, típicamente se cuantifica midiendo el **potencial hídrico** de la hoja (Ψ). El Ψ de la hoja es uno de los indicadores más fiables del estatus hídrico de la planta y su medida proporciona un dato para el estudio de las respuestas de la planta a la sequía (Boyer 1995). Bajo condiciones de riego óptimo, la micorrización tiene un pequeño papel en la alteración del Ψ de la hoja de muchas especies vegetales (Bryla y Duniway 1997; Davies *et al.* 1993; Ebel *et al.* 1994). Sin embargo, en condiciones de estrés hídrico, la simbiosis MA modifica el Ψ. Subramanian *et al.* (1995) mostraron que las plantas MA de maíz tuvieron un valor de Ψ más elevado (menos negativo) a lo largo de 3 semanas de sequía. Tras este tratamiento de sequía, las plantas no MA tuvieron un Ψ de –2,5 MPa, mientras que las plantas MA mantuvieron un Ψ de –1,7 MPa, indicando su capacidad de resistir las condiciones de déficit hídrico.

La colonización micorrícica también puede influir en el comportamiento de los estomas de la hoja de plantas hospedadoras disminuyendo la resistencia estomática bajo condiciones de sequía y recuperación (Fitter 1988; Ruiz-Lozano *et al.*, 1995; Duan *et al.*, 1996). Algunos autores han propuesto que los valores más bajos de resistencia estomática en plantas MA que en las plantas no MA se debían a efectos nutricionales del P (Fitter 1988, Koide 1985; Goicoechea *et al.*, 1997). Otros sugieren que la mejora estomática está influenciada por cambios hormonales en plantas MA (Allen 1982, Danneberg *et al.*, 1992; Duan *et al.,* 1996) también sugirió que el hongo MA incrementa la capacidad del sistema radical de tomar agua de suelos más secos lo que conlleva una menor resistencia estomática bajo condiciones de sequía.

De igual modo se ha observado que las plantas micorrizadas incrementan la tasa fotosintética y presentan una mayor eficiencia en el uso de agua (cantidad de CO_2 fijado en la fotosíntesis por mol de agua transpirado) (Ruiz-Lozano *et al.*, 1995, 1996a; Ruiz-Lozano y Azcón, 1995), efectos, probablemente mediados por cambios en el balance hormonal de las plantas (Duan *et al.*, 1996; Goicoechea *et al.*, 1997).

4.5. Estimulación de actividades asimilativas esenciales para la planta

Entre estas actividades destaca la nitrato reductasa (NR), que es el primer enzima implicado en el proceso de asimilación del nitrato, el cual es, a su vez, la principal fuente de nitrógeno para la mayoría de las plantas superiores (Azcón *et al.*, 2001). La reacción catalizada por dicho enzima representa, probablemente, el paso

limitante en el proceso de asimilación (Campbell, 1988). Sin embargo, la NR es un enzima que resulta fuertemente afectada por la sequía, disminuyendo drásticamente su actividad (Sánchez-Díaz y Aguirreolea, 1993). Los estudios realizados hasta la fecha, han demostrado que los hongos MA poseen la información genética que codifica para la NR (Kaldorf *et al.*, 1994) y que la actividad NR se localiza en arbúsculos de células colonizadas (Kaldorf *et al.*, 1998). Además, también se ha demostrado que la simbiosis MA permite mantener niveles de actividad NR más elevados en las plantas micorrizadas y sometidas a estrés hídrico que en las controles sin micorrizar, contribuyendo de esta forma a incrementar la tolerancia de las plantas frente a los efectos negativos de la limitación hídrica (Tobar *et al.*, 1994; Ruiz-Lozano y Azcón, 1996).

Las actividades de muchos de los enzimas tienden a ser más altas en plantas de maíz MA que en las no MA. Esto también ocurre con la glutamina sintetasa (GS) y la glutamato sintasa (GOGAT), que en raíces MA muestran un incremento del 45-75% en comparación con las raíces no MA bajo condiciones de sequía. Esto sugiere dos posibilidades: bien que el hongo MA induzca un incremento de dichas actividades enzimáticas en raíces, o bien que las estructuras fúngicas tengan estas actividades enzimáticas. Johansen *et al.* (1996) informó de la presencia de un sistema funcional GS-GOGAT en el micelio extrarradical. Toussaint *et al.* (2004) han identificado dos monómeros GS diferentes en el micelio extrarradical de raíces de zanahoria. Govidarajulu *et al.* (2005) también mostraron que el micelio extrarradical asimila la mayoria del ^{15}N en aminoácidos (arginina, ácido aspártico, asparagina y glutamina) para ser luego transferidos a las raíces hospedadoras.

Todo ello sugiere que la cantidad de N que es activamente asimilado en el micelio extrarradical tiene un gran impacto en el metabolismo nitrogenado de la planta hospedadora y parece ser un mecanismo potencial relacionado con la tolerancia al estrés hídrico.

4.6. Protección frente al daño oxidativo generado por la limitación hídrica

La sequía causa un estrés oxidativo en plantas y muchas de las reacciones degenerativas asociadas con estreses abióticos están mediadas por especies reducidas del oxígeno tales como radicales superóxido o peróxido de hidrógeno (Smirnoff, 1993).

Hasta la fecha, la información existente sobre actividades enzimáticas antioxidantes en la simbiosis MA es escasa y se ha centrado en el papel de las superóxido dismutasas (SODs), mientras que no hay estudios sobre otros enzimas implicados en la eliminación de especies reactivas del oxígeno como catalasas, peroxidadas o glutation reductasas. Así, los estudios pioneros demostraron que el hongo MA *Glomus mosseae* posee actividad CuZn-SOD y que las raíces de trébol micorrizadas presentan isoformas nuevas de CuZn-SOD y de Mn-SOD respecto a los controles sin micorrizar (Palma *et al.*, 1993). Con posterioridad, estudios llevados a cabo por Ruiz-Lozano et al., (1996), demostraron que plantas de lechuga micorrizadas y sometidas a estrés hídrico presentaban niveles de actividad SOD superiores a los correspondientes controles sin micorrizar. Estos resultados se complementan con

otros más recientes en los que se consiguió clonar varios cDNAs que codifican SODs. Uno de los genes clonados (*Mn-sod II*) resultó estar sobre expresado específicamente en las raíces de las plantas micorrizadas a consecuencia de la limitación hídrica (Ruiz-Lozano *et al.*, 2001a). Tanto el incremento en la actividad SOD (Ruiz-Lozano *et al.*, 1996b) como el incremento en la expresión del gen *Mn-sodII* (Ruiz-Lozano *et al.*, 2001a) estaban relacionados con la mejora en la tolerancia a la sequía, por la simbiosis MA.

Estudios, como los realizados en plantas de soja sometidas a estrés hídrico, han mostrado una considerable disminución del daño oxidativo a lípidos y proteínas en nódulos respecto a los correspondientes controles sin micorrizar (Ruiz-Lozano *et al.*, 2001b). Dicha disminución del daño oxidativo a biomoléculas parece ser el mecanismo más importante a través del cual la simbiosis MA protegió a las plantas frente al fenómeno de senescencia nodular prematura inducida por la limitación hídrica. Además, la simbiosis MA puede incrementar considerablemente la actividad GR tanto en raíces como en nódulos de plantas de soja sometidas a estrés hídrico (Porcel *et al.*, 2003). La GR es un componente importante del ciclo del ascorbato-glutation ya que es el enzima que regenera el glutation oxidado en su forma reducida (Noctor y Foyer, 1998).

Todos estos resultados sugieren que la protección de la micorriza frente al estrés oxidativo causado por sequía puede ser uno de los mecanismos más importantes por los cuales la simbiosis MA incrementa la tolerancia de las plantas hospedadoras a la sequía. Estas observaciones concuerdan con la propuesta de Bartels (2001) que indica que, tanto la prevención del estrés oxidativo como la eliminación de especies reactivas del oxígeno, son las tácticas más efectivas usadas por las plantas para conseguir la tolerancia frente a muchos estreses abióticos, incluyendo la sequía.

4.7. Cambios nutricionales en las plantas hospedadoras

Una de las funciones mejor conocida de la simbiosis MA es la adquisición de nutrientes con baja movilidad en el suelo. Se ha visto que la simbiosis MA es importante para la nutrición del P en la planta. La porción disponible de P total del suelo, normalmente menor del 1%, está controlada principalmente por reacciones químicas y procesos biológicos. Además, el nivel de difusión de iones PO_4^- en suelo es extremadamente bajo (10^{-8}–10^{-11} cm^{-2} s^{-1}) y varía con la humedad y el contenido de P del suelo. Se ha demostrado que la asociación MA mejora la captación de P del suelo en sequía (Nelsen y Safir, 1982; Sylvia *et al.*, 1993; Ruiz-Lozano *et al.*, 1995a) y en condiciones óptimas (George *et al.*, 1995; Hetrick *et al.*, 1996; McArthur y Knowles, 1993). La mejora en la captación de P por las plantas MA parece ser debida principalmente al micelio extrarradical (MER), que absorbe P de la solución del suelo y lo transfiere a las raíces. Estos procesos son mucho más rápidos que la difusión de P en el suelo solamente (Jakobsen *et al.*, 1992). Ya que el MER contribuye más del 70% en el estatus de P de la planta hospedadora (George *et al.*, 1994), la hifa MA tiene la capacidad de satisfacer casi completamente la demanda de P de las plantas suministrándolo de la parte del suelo que está inexplorada por las raíces. Como resultado de un incremento en la captación de P en la raíz, las

plantas MA producen con frecuencia cosechas mayores que las plantas no MA (Smith y Read 1997). La correlación entre el nivel de P en la planta y el Ψ de la hoja sugiere que la nutrición del P mejorada por la simbiosis MA es una hipótesis convincente para la tolerancia a la sequía de plantas (Subramanian *et al.*, 1997). Por el contrario, Augé *et al.,* (1994) y Davies *et al.*, (1993) afirmaron que la tolerancia a la sequía en plantas era independiente de la captación de P estimulada por el hongo MA.

El nitrógeno es considerado el nutriente esencial más limitante requerido para el crecimiento de la planta en ecosistemas tropicales. Como la forma de asimilar el N en cualquier cultivo es NO_3^-, altamente lábil en suelo, el papel de la micorriza se ha considerado insignificante. Sin embargo, el estrés hídrico impide la movilidad de los iones NO_3^- en suelo debido a su bajo nivel de concentración y difusión (Azcón *et al.* 1996). Bajo condiciones óptimas, el hongo MA tiene un papel crucial en el transporte de N del suelo a la raíz, contribuyendo así al crecimiento y nutrición de la planta. Los estudios de radioisótopos han revelado que el micelio extrarradical puede obtener ^{15}N (NO_3^- o NH_4^+) del suelo (Frey y Shüepp, 1993; Johansen *et al.*, 1994, 1996), o en sistemas de raíz de zanahoria in vitro (Villegas *et al.*, 1996; Toussaint *et al.*, 2004: Govindarajulu *et al.*, 2005). De esta forma, las plantas MA tienen acceso a formas del N que no están disponibles para las plantas no MA (Azcón-Aguilar *et al.*, 1993; Tobar *et al.*, 1994a y b; Azcón *et al.*, 2003).

El potasio, catión soluble responsable del movimiento de los estomas (Premachandra *et al.*, 1993), puede tener una función en la tolerancia a la sequía en plantas. Los efectos de la micorriza sobre la nutrición del K aún no han sido estudiados profundamente. Smith *et al.* (1981) observaron elevadas concentraciones de K en las raíces MA de trébol cuando crecían en suelos deficientes de P. Cuando a las plantas no MA se les suministró P suficiente, el nivel de K de plantas MA y no MA fue similar, sugiriendo que el incremento en el K era debido a un efecto indirecto del P. Ruiz-Lozano *et al.* (1995) observaron que la protección de las plantas MA de lechuga frente al estrés hídrico se debía en parte al incremento en la captación del K.

El nivel de micronutrientes de suelos tropicales es normalmente bajo y los suelos cultivables a menudo son deficientes en estos nutrientes. Se ha visto que la colonización de la raíz por un hongo MA mejora la productividad de estos suelos aumentando la captación de micronutrientes de difusión lenta como el Cu o el Zn (Sylvia *et al.*, 1993). Un gran número de estudios han mostrado que la captación de Zn vía micorriza es importante para la mejora de la deficiencia de Zn (Evans y Miller 1988, Sylvia *et al.*, 1993). Bajo condiciones de estrés hídrico, la disponibilidad de nutrientes para la planta está muy restringida debido a la movilidad nula de los iones minerales. Consecuentemente, el crecimiento de plantas no MA está limitado por la disponibilidad de nutrientes, y el crecimiento reducido de la raíz frenaría la accesibilidad de agua. Bajo estas condiciones, la contribución de la micorriza a la captación de nutrientes y/o agua sería de gran importancia.

1. MATERIAL BIOLÓGICO Y CONDICIONES DE CRECIMIENTO

1.1. Material vegetal

1.1.1. Plantas utilizadas

Las plantas utilizadas en este trabajo fueron soja (*Glycine max* L. cv Williams), lechuga (*Lactuca sativa* L. cv Romana), maíz (*Zea mays* L. cv Prisma) y tabaco (*Nicotiana tabacum* L. cv Samsun), ésta última con dos líneas de plantas, una mutante antisentido para el gen *NtAQP1* y la correspondiente línea parental de fenotipo salvaje.

1.1.2. Cultivo de plantas

1.1.2.1. Substrato: composición y esterilización

El substrato en todos los experimentos estaba compuesto por una mezcla de suelo:arena en proporción 1:1 (v/v).

El suelo utilizado procedía de la Estación Experimental del Zaidín (CSIC-Granada). Presentaba las siguientes características: pH de 8.1 (agua); 1.81% materia orgánica, concentraciones de nutrientes (mg kg^{-1}) N, 2.5; P, 6.2 (extraíble con $NaHCO_3$); K, 132.0. La textura del suelo estaba formada por 35.8% arena, 43.6% limo y 20.5% arcilla.

La arena de cuarzo (< 2 mm) previamente era lavada y esterilizada en autoclave 20 minutos a 120º C. El suelo era tamizado (2 mm) y esterilizado por tindalización en un autoclave a vapor fluente (100°C durante 1 h, 3 días consecutivos). De esta forma, queda libre de propágulos de micorrizas y otros organismos que puedan interferir en el experimento.

1.1.2.2. Esterilización, germinación y siembra de las semillas

Las **semillas de soja** fueron esterilizadas en superficie con una solución de H_2O_2 al 15% durante 8 minutos. Luego se lavaron varias veces con agua estéril para eliminar cualquier resto que pudiera interferir en la germinación. Posteriormente las semillas de soja germinaron en vermiculita estéril a 25º C durante aproximadamente 3 días, momento en el que se transplantaron a macetas que contenían 600 g de una mezcla de suelo:arena estéril.

Las **semillas de lechuga** se colocaron directamente en macetas para su germinación en la maceta definitiva.

Las **semillas de tabaco** fueron esterilizadas en superficie con etanol al 70% durante 2 minutos y con hipoclorito de sodio al 2,5% durante 10 minutos. Posteriormente, se lavaron varias veces con agua estéril para eliminar posibles restos. Tras la esterilización, las semillas de tabaco de fenotipo salvaje se colocaron en medio MS 1/2 (Murashige y Skoog, 1962), mientras que las semillas de tabaco mutantes se colocaron en el mismo medio adicionado con kanamicina (100 µg/ml) para la selección de plantas mutantes antisentido (Siefritz *et al.*, 2002). Después de 10 días de incubación a 25º C, las semillas se transfirieron a macetas con 500 g de una mezcla de suelo:arena estéril.

1.1.2.3. Solución nutritiva para las plantas

Las plantas de soja recibieron durante todo su crecimiento un aporte semanal de 10 ml de solución nutritiva Hewitt sin N ni P (Hewitt, 1952). Los tratamientos recibieron a las 3 semanas de la siembra, la misma solución nutritiva adicionada con 0.18 mM H_2HPO_4 y 2 mM NH_4NO_3 (plantas NI), 0.35 mM H_2HPO_4 (plantas Br), 3 mM NH_4NO_3 (plantas Gm y Gi), con el objetivo de tratar de conseguir plantas con tamaño y contenido en nutrientes similares en todos los tratamientos.

Las plantas de tabaco no recibieron ningún tipo de solución nutritiva.

Las plantas de lechuga no inoculadas recibieron cada semana un aporte de 10 ml de solución nutritiva Hewitt (Hewitt, 1952), modificada para contener 4 mM N + 1 mM P. Las plantas micorrizadas no recibieron solución nutritiva.

La composición de la solución nutritiva es la siguiente:

Compuesto	Concentración (µM)	Solución madre (g/l)	Para 1 litro (ml)
KNO_3	3×10^3	30,3	10
$Ca(NO_3)_2 \cdot 4H_2O$	9×10^3	101,54	20
$NaH_2PO_4 \cdot 2H_2O$	300	18,4	2,5
$MgSO_4 \cdot 7H_2O$	150	18,4	20
EDTA-Fe	67	2,45	10
$MnSO_4 \cdot 7H_2O$	13	1,35	1
$CuSO_4 \cdot 5H_2O$	1	2,4	0,1
$ZnSO_4 \cdot 2H_2O$	1	4,22	0,1
H_3BO_3	30	18,6	0,1
$Na_2MoO_4 \cdot 2H_2O$	0,15	0,35	0,1

Ajustar a pH 7

1.1.2.4. Condiciones de crecimiento en cámara de cultivo

Todas las plantas crecieron en condiciones controladas en una cámara de cultivo con un 70-80% de humedad relativa, temperaturas día/noche de 25/15º C, un fotoperíodo de 16 horas y una intensidad luminosa de 460 µmol m^{-2} s^{-1} (Li-Cor, Lincoln, NE, USA, modelo LI-188B).

1.1.2.5. Aplicación del déficit hídrico

El contenido de agua en el suelo se controló con un dispositivo ThetaProbe ML2 (AT-Delta-D Device, Ltd, UK), que mide el contenido volumétrico en agua del suelo en base a cambios en la constante dieléctrica del suelo cuando está húmedo. El contenido volumétrico de agua en un suelo es un cociente entre el volumen de agua presente en el suelo y el volumen total de la muestra de suelo, por lo que es un parámetro adimensional expresado como % vol (Roth *et al.*, 1992). En todos los casos, la mitad de las plantas que constituían el experimento se sometió a déficit hídrico mientras que la otra mitad recibió un aporte hídrico óptimo. El agua se suministró diariamente para mantener constante la humedad del suelo cercana a su capacidad de campo (17% humedad volumétrica del suelo) durante las primeras 5 semanas del crecimiento de las plantas. En este momento, la mitad de las plantas se llevaron a un 70% de su capacidad de campo (generalmente durante 3 días), lo cual correspondía al 10% de la humedad volumétrica del suelo. Las plantas se mantuvieron en estas condiciones durante 10 días más. Para controlar el nivel de estrés hídrico, el contenido de agua del suelo se midió diariamente

con el ThetaProbe ML2 (al final de la tarde) y la cantidad de agua perdida se añadió a cada maceta para reponer el contenido de agua al 10% de la humedad volumétrica del suelo (70% de la capacidad de campo).

1.2. Material fúngico

1.2.1. Hongos MA utilizados

Las especies de hongos MA utilizados fueron *Glomus mosseae* (Nicol. y Gerd.) Gerd. Y Trappe, aislado EEZ 6, BEG 122 y *Glomus intraradices* (Schenck y Smith) aislado BEG 121. El inóculo de la micorriza de cada endofito creció en cultivos de *Allium cepa* L. o *Zea mays* . En ambos casos estaba formado por suelo, esporas, micelio y fragmentos de raíz infectados.

1.2.2. Determinación de la micorrización

Una vez obtenidas en el momento de la cosecha, las raíces fueron teñidas siguiendo el método de Phillips y Hayman (1970). El colorante azul tripán tiñe las estructuras que contienen quitina, principal componente de las pareces celulares de algunos hongos, entre ellos los formadores de micorrizas arbusculares (Bartinicki-García, 1968). Se observan así los componentes del hongo en el interior de la raíz sin que ésta se coloree.
Procedimiento:

1. Las raíces se trocean y se sumergen en una solución de KOH 10% (p/v) y se mantienen durante 20 min al *baño maría.*
2. Se elimina el KOH lavando varias veces las raíces con agua corriente.
3. Se les añadió HCl 0,1 N durante 2 min con el fin de preparar las raíces para la entrada del colorante.
4. De nuevo se ponen a hervir al baño María en una solución de 0,05% azul tripán en ácido láctico (v/v) durante 15-20 min.
5. Una vez teñidas, se elimina el exceso de colorante y se conservan en una solución de ácido láctico hasta su posterior observación al microscopio óptico.

1.2.3. Estimación de la colonización micorrícica

El grado de colonización micorrícica se midió usando el método de Giovanetti y Mosse (1980). Para ello, se colocan en portaobjetos fragmentos elegidos al azar de aproximadamente 1 cm de raíz y se cuantifican los parámetros de la micorrización. Cuando era necesaria una evaluación más exhaustiva del desarrollo del hongo en la raíz de la planta se siguió el método de Trouvelot *et al.* (1986) asignando a cada fragmento un número de 0 a 5 en función del nivel de infección por el hongo, así como un valor A0 hasta A3 en base a la riqueza de arbúsculos del fragmento estudiado.

Con posterioridad un programa informático calcula la frecuencia de colonización, la intensidad de la misma y la riqueza de arbúsculos (Para más información, visitar la web http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html)

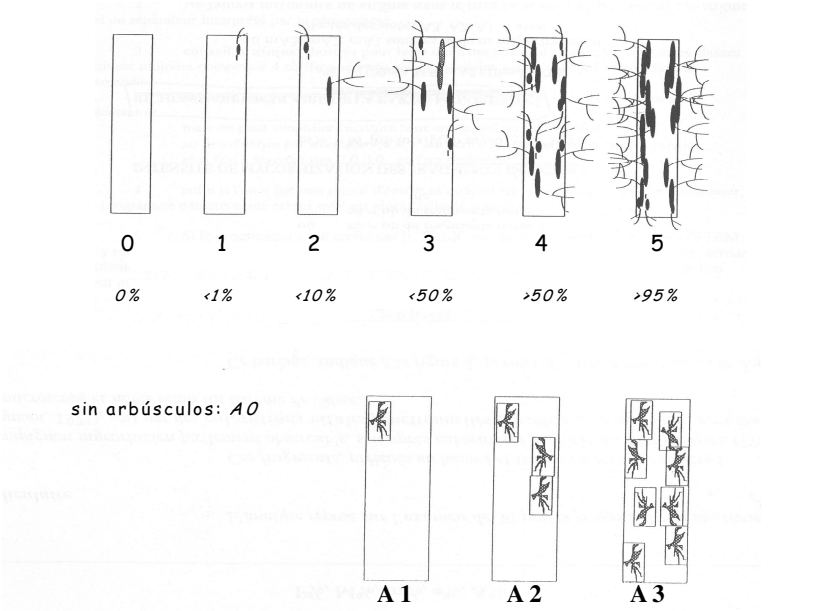


Figura 1. Método de Trouvelot

1.2.4. Cultivo monoxénico de hongos MA

La construcción de una genoteca de ADNc de G. intraradices se hizo a partir de un cultivo de este hongo en un sistema monoxénico y en plantas de Petri divididas en dos compartimentos según el método descrito por St. Arnaud (1996). El medio de cultivo monoxénico de los hongos MA se estableció en medio M con sacarosa (10 g/l) como fuente de carbono en un compartimento y medio M sin fuente de carbono (Bago y Cano, 2005) en el otro compartimento. Su composición es la siguiente:

1.21 mM $Ca(NO_3)_2$
21.8 mM NaFe/EDTA
4.51 μM KI
10% solución de macroelementos
0.1% solución de microelementos
1% vitaminas
1% sacarosa
0.35% Phytagel

El pH se ajustó a 5.5 con NaOH y el medio se esterilizó en el autoclave a 120 °C durante 20 min.

Solución de Macroelementos: 30 mM $MgSO_4$, 7.9 mM KNO_3, 8.7 mM KCl, 0.35 mM KH_2PO_4, 1.4 mM NaH_2PO_4, 14 mM $NaSO_4$.
Solución de Microelementos: 30 mM MnCl2, 9.3 mM ZnSO4, 24.2 mM H_3BO_3, 5 mM CuSO4, 10 μM Na2MoO4.

Vitaminas (100x): 4 mM glicina, 29.6 μM hidrocloruro de tiamina, 49 μM hidrocloruro de piridoxina, 0.4 mM ácido nicotínico, 27.7 mM mio-inositol.

1.3. Material bacteriano

1.3.1. Bacterias utilizadas

Bradyrrhizobium japonicum strain USDA 110.

Fue utilizada en los experimentos donde las plantas de soja se inocularon con una bacteria fijadora de nitrógeno.

Escherichia coli cepa XL1-Blue.

Es una bacteria que permite transformaciones altamente eficientes y la selección de clones recombinantes mediante detección de actividad B-galactosidasa. Fue utilizada para la preparación de células quimiocompetentes para transformación con plásmidos que contuvieran insertos de genes de interés y para la obtención del ADN plasmídico.

1.3.2. Medios de cultivo

A) Medio PSY líquido para *Bradyrrhizobium japonicum* en cultivo líquido.

KH_2PO_4	0,3 g/l
K_2HPO_4	0,3 g/l
$CaCl_2$ $2H_2O$	0,005 g/l
$MgSO_4$ $7H_2O$	0,1 g/l
Peptona	3 g/l
Extracto levadura	1 g/l
Solución minerales	10 ml/l (stock 100x)
Solución vitaminas	1 ml/l (stock 1000x)
L-arabinosa 10%	0,1% vol. final
Cloranfenicol	15 μg/ml de concentración final
H_2O	hasta 1 litro

Solución de minerales (100x): HBO_3 1 g/l; $ZnSO_4$ 7 H_2O 0,1 g/l; $CuSO_4$ 5 H_2O 0,05 g/l; $MnCl_2$ 4 H_2O 0,05 g/l; $NaMoO_4$ 2 H_2O 0,01 g/l; $FeCl_3$ xhidratado 0,1 g/l

Solución de vitaminas (1000x): Tiamina 0,1 g/l; Biotina 0,1 g/l; ácido pantoténico o pantotenato sódico 0,1 g/l

Notas:

- Autoclavar por filtración las soluciones de vitaminas y L-arabinosa y adicionar al medio junto con el cloranfenicol después de estar autoclavado.
- Ajustar el medio a pH 7 y autoclavar 20 minutos a 120 ºC

B) Medio LB (*Luria-Bertani*) para *Escherichia coli*

Triptona	10 g/l
Extracto de levadura	5 g/l
NaCl	10 g/l

Agua destilada hasta 1 litro

Ajustar el pH a 7.0 con NaOH 5N
Autoclavar 20 min a 120 °C

1.3.3. Mantenimiento de las cepas

Las cepas de *B. japonicum* y *E. coli* descritas anteriormente se conservaron a –80 °C en glicerol al 15%. Para ello se cultivan en medio líquido selectivo hasta el final de la fase de crecimiento exponencial (cultivo de 16 h). A 800 µl de estos cultivos se le añaden 400 µl de glicerol al 50% estéril, se mezcla bien y se almacenan a –80 °C.

1.3.4. Marcadores de selección

Como marcadores de selección de las colonias recombinantes se utilizaron soluciones de IPTG y X-gal, conservadas a –20 °C.

IPTG (isopropil-beta-D-tiogalactopiranósido) se usa para inducir la expresión del gen LacZ en *E.coli.*

La solución de trabajo se prepara a una concentración de 20 mg/ml, se esteriliza por filtración y se guarda a –20 °C. Se añaden 40µl a la placa unos 30 min antes de sembrar.

X-gal (5-bromo-4-cloro-3-indolil-beta-D-galactopiranósido) es un sustrato cromogénico para la β-galactosidasa de modo que al ser hidrolizado forma precipitados azules.

El X-gal se prepara en dimetilformamida a una concentración de 20 mg/ml. Se debe proteger de la luz debido a su fotosensibilidad y no es necesario filtrar. Se añaden 40µl a la placa unos 30 min antes de sembrar.

En el "screanning" se usan tanto el IPTG como el X-gal para diferenciar colonias recombinantes (blancas) de las no recombinantes (azules).

1.3.5. Preparación de células quimiocompetentes

La preparación de células competentes de *E.coli* se realizó según la técnica descrita por Hanahan (1983).

El procedimiento es el siguiente:

1. Inocular 2 ml de un precultivo de *E.coli* en 50 ml de medio LB suplementado con 10 mM de $MgSO_4$.
2. Incubar a 37 °C hasta alcanzar una DO_{550} de 0,5 (1-2 horas).
3. Transferir el cultivo a tubos de centrífuga SS-34 y enfriar en hielo 15 min.
4. Centrifugar los tubos a 800 g durante 12 min a 4 °C.
5. Retirar sobrenadante suavemente y secar bien las paredes del tubo.
6. Resuspender las células en 16 ml de solución RFI y mantener durante 15 min en hielo.
7. Centrifugar como en el paso 4 y resuspender las células en 2 ml de RFII e incubar 15 min en hielo.
8. Hacer alícuotas de 100 µl.
9. Enfriar los eppendorf rápidamente en N_2 líquido y congelar a –80 °C.

Todo el material usado (puntas, tubos de centrífuga, eppendorf…) debe estar estéril y muy frío.

RFI: 100 mM RbCl, 50 mM $MnCl_2$, 10 mM acetato potásico, 10 mM $CaCl_2$, 11,9% Glicerol. Ajustar a pH 5.8 con ácido acético 0,2 M. Esterilizar por filtración.
RFII: 10 mM MOPS, 1 mM RbCl, 100 mM $CaCl_2$, 11,9% Glicerol. Ajustar a pH 6.8 con NaOH 0,5 M. Esterilizar por filtración.

2. TÉCNICAS BIOQUÍMICAS Y DETERMINACIONES ENZIMÁTICAS

2.1. Técnicas bioquímicas

2.1.1. Extracción de proteínas totales

La extracción de proteínas totales se hizo según el método descrito por Daniels *et al.* (1994) con alguna modificación.
Procedimiento:

1. La muestra congelada se homogeniza en N_2 líquido con un mortero y un pistilo hasta conseguir una textura polvo.
2. Por cada gramo de muestra se añaden 5 ml de tampón de extracción. Se le da un vórtex y se mantiene en hielo.
3. Centrifugar a 20 000 g durante 20 min a 4 °C.
4. Recoger el sobrenadante y completar con tampón de extracción hasta llenar el tubo de la ultracentrífuga.
5. Ultracentrifugar 2 horas a 4 °C y 100 000 g y resuspender el pellet resultante en 20-50 μl de SDS 0,1% (p/v) preparado en el tampón de extracción.

Tampón de extracción: 10 mM Tris pH 7.5; 1 mM EDTA; 12% (p/v) sacarosa; 0.2 mM PMSF (inhibidor de proteasas); 2 μg/ml aprotinina; 1 μg/ml leupeptina.
Notas: la solución de PMSF se prepara en etanol al 96% (p/v) y se conserva a 4 °C

2.1.2. Cuantificación de proteínas solubles

La cuantificación de proteínas totales solubles se llevó a cabo mediante el método de Bradford (1976) que permite determinar espectrofotométricamente el contenido de proteínas mediante la medida del complejo que éstas forman con un colorante. Se empleó un reactivo comercial de Bio-Rad "Dye Reagent Protein Assay", y albúmina de suero bovina (BSA) como proteína de referencia para preparar la curva patrón de proteínas (0, 2, 4, 6, 8, 10, 12, 14, 16, 18 y 20 μg BSA ml^{-1}). Se adicionaron 200 μl de reactivo Bio-Rad a 800 μl de un volumen compuesto por la muestra o BSA, convenientemente diluidas, y agua destilada, y se midió la absorbancia a 595 nm, de 5 min a 1 h tras la adición del reactivo (período de estabilidad del complejo). Conocidos los valores de absorbancia, se elaboró una recta de regresión lineal con los datos de la curva patrón de albúmina y se calculó la concentración de proteínas interpolando los valores de absorbancia de las muestras.

2.1.3. Gel desnaturalizante de poliacrilamida

La separación electroforética se realizó en un equipo de electroforesis vertical Mini-Protean (Biorad, USA) para geles de 8 cm de ancho x 7cm de longitud x 1 mm de espesor. Las proteínas (10μg) se solubilizaron hirviéndolas 4 min en tampón de carga. Las proteínas solubilizadas y las proteínas patrón de peso molecular (Pre Stained SDS Standard Broad Range de Biorad, USA) Se separaron en un gel de poliacrilamida (2 cm de gel de concentración y 5 cm de gel de separación al 15% de acrilamida) a corriente constante (20 mA/gel) durante una hora aproximadamente.

Tampón de carga (6x):120 mM Tris-HCl pH 8.6; 6% SDS; 2% β-mercaptoetanol; 50% glicerol; 1% azul de bromofenol.
Tampón de electroforesis (10x): Tris 30 g/l; Glicina 144 g/l; SDS 10 g/l. Ajustar el pH a 8.3 con HCl.
Tampón concentrador: 0.5 M Tris-HCl pH 6.8; 0.4% SDS
Tampón separador: 1.5 M Tris-HCl pH 8.8; 0.4% SDS

Componentes	Gel concentrador	Gel separador
Tampón concentrador	1.3 ml	---------
Tampón separador	--------	2.5 ml
Acrilamida 40%	0.5 ml	3.6 ml
Bis-acrilamida 2%	0.3 ml	2 ml
H_2O-destilada	2.9 ml	1.9 ml
APS 10%	60 μl	160 μl
TEMED	20 μl	15 μl

2.1.4. Tinción de las proteínas en los geles

Una vez terminada la electroforesis, los geles se lavan varias veces con H_2O-destilada durante 15 minutos agitando a temperatura ambiente. Luego se tiñen para la visualización de las proteínas con 5 ml del reactivo GelCode® Blue Stain Reagent (Pierce, IL, USA). Se ponen a agitar durante 1 hora a temperatura ambiente. Posteriormente se vuelven a lavar como antes, con H_2O-destilada para eliminar el exceso de colorante.

2.1.5. Western blot

2.1.5.1. Transferencia de proteínas a membrana

La transferencia se realizó siguiendo el método de Towbin *et al.* (1979) en una cubeta de transferencia Transblot (Biorad, USA). La metodología empleada es la siguiente:

1. Cortar una membrana Immun-Blot™ PVDF Membrane (Biorad, USA) y 2 hojas de papel absorbente (Whatman 3MM o equivalente) del tamaño del gel.
2. Humedecer la membrana en metanol absoluto durante 1-2 min.
3. Ensamblar el sistema sumergiendo todo en tampón de transferencia siguiendo este orden de abajo a arriba: esponja, Whatman, gel, membrana, Whatman y esponja.

4. Colocar el sándwich en el tanque de transferencia con la membrana lo más cerca posible del electrodo positivo.
5. Transferir durante 2 h a 100-110 mA en tampón de transferencia a 4 °C.

Tampón de transferencia: Tris 3.63 g/l; Glicina 14.4 g/l; SDS 0.37 g/l; metanol 200 ml/l. Mantener a 4°C hasta el momento de uso.

2.1.5.2. Inmunodetección

El procedimiento para la inmunodetección fue el siguiente:

1. Añadir a la membrana 10-15 ml de TTBS con el anticuerpo primario adicionado (Anti PIP1 de pollo) diluido 1:750.
2. Agitar 1 h a temperatura ambiente o bien toda la noche a 4 °C
3. Tirar la solución y lavar 3 veces 5 min con TTBS.
4. Añadir el anticuerpo secundario (Anti-chicken de ratón) en dilución 1:10000. Agitar 1 hora a temperatura ambiente.
5. Mezclar 7 ml de cada solución del kit comercial SuperSignal® West Pico Chemiluminiscent Substrate (Pierce, IL, USA)
   y añadir a la membrana. Agitar 1-5 min a temperatura ambiente.
6. Secar la membrana con papel absorbente e incubar poniendo la membrana envuelta en film transparente junto a una película Hyperfilm ECL (Amersham, EE.UU.) durante 30 seg-1 h.
7. Revelar la película normalmente.

TTBS 0.05%: 250 ml tween 20% en 500 ml de TBS 1x.
TBS 10x: Tris pH 7.5 30 g/l; NaCl 80 g/l; KCl 2 g/l.
Tampón de bloqueo: 5% leche en polvo desnatada en TTBS al 0.05%

2.2. Determinaciones enzimáticas del metabolismo oxidativo

2.2.1. Obtención de los extractos enzimáticos

Los enzimas se extrajeron de aproximadamente 1 gr de peso fresco de hoja raíz a 0-4 °C. El procedimiento fue el siguiente:

1. Homogeneizar el material de partida congelado en un mortero con N_2 líquido y un medio de extracción adicionado con 50 mg PVPP (evita daños por fenoles y quinonas). Se usan 10 ml de medio por gramo de material de partida.
2. Transferir en polvo resultante a tubos estériles de centrífuga. Agitar y mantener en hielo.
3. Filtrar la muestra por 4 gasas de nylon.
4. Centrifugar a 20 000 g, 20 min a 0-4 °C.
5. Alicuotar y mantener a –80 °C hasta la medida enzimática.

Medio extracción para SOD y CAT	Medio extracción para APX	Medio extracción para GR
Tampón PK 50 mM pH 7.8	**Tampón PK 50 mM pH 7.8**	**Tampón PK 50 mM pH 7.8**
EDTA 0.1 mM	**EDTA 0.1 mM**	**EDTA 0.1 mM**
PVPP 1% (p/v)	**PVPP 1% (p/v)**	**PVPP 1% (p/v)**
	Ácido ascórbico 4 mM	**β-mercaptoetanol 10 mM**

2.2.2. Actividad superóxido dismutasa (SOD; EC 1.15.1.1.)

La actividad SOD se midió según Beyer y Fridovich (1987) basado en la capacidad del SOD de inhibir la reducción del nitroblue tetrazolium (NBT) por los radicales superóxido generados fotoquímicamente. Una unidad de SOD se define como la cantidad de enzima requerido para inhibir la reducción del NBT un 50% a 25 °C.
El procedimiento es el siguiente:
La mezcla de reacción, en un volumen de 4,1 ml contiene:

Riboflavina 1.3 μM
Metionina 13 mM
NBT 63 mM
Tampón Na_2CO_3-$NaHCO_3$ 50 mM, pH 10.2, EDTA 0,1 mM

Se utilizan tubos de vidrio de grosor y color uniforme que se colocan en una gradilla de plástico circular, sumergida en un baño de vidrio aclimatado a 25 °C. Una lámpara fluorescente circular (Osram-L 40W/10C) rodea toda la pared del baño, cerrándose todo el conjunto con una tapadera recubierta de papel de aluminio. La reacción se inicia encendiendo la lámpara. Como blancos se utilizan soluciones idénticas que no son iluminadas. Se mide la D.O. a 560 nm de longitud de onda.

	Tampón-Rib-Met-NBT (ml)	muestra (ml)	
CMC (control máxima coloración)	4	_____	ILUMINACIÓN
Problema	4	0.1	ILUMINACIÓN
Blanco	4	0.1	_____

Los tiempos de iluminación empleados son 100 segundos. En presencia de SOD se inhibe la reacción, calculándose el % de inhibición producido.
Unidades SOD/ml = (%inhibición/50) / factor dilución

$$A_{550/min} = A_{550/min}\ (problema) - A_{550/min}\ (blanco)$$

$$\text{Unidades SOD/ml} = \frac{\%\ \text{inhibición}}{50\%} \times \frac{1}{\text{volumen de muestra (ml)}} \times \text{factor de dilución}$$

$$\% \text{ inhibición} = \left(\frac{A_{550/min}\,(CMC) - A_{550/min}}{A_{550/min}\,(CMC)} \right) \times 100$$

2.2.3. Actividad catalasa (CAT; EC 1.11.1.6.)

Se siguió el método espectrofotométrico descrito por Aebi (1984), basado en la medida de la disminución de la absorbancia del H_2O_2 a 240 nm debida a su descomposición por efecto de la catalasa. En una cubeta de cuarzo se añadieron 5-100 µl de muestra a 2.9 ml de mezcla de reacción formada por tampón fosfato-K 50 mM, pH 7 y H_2O_2 10.6 mM, convenientemente protegida de la luz. La reacción se registró durante 2 min a 25 ºC en un espectrofotómetro Beckman modelo DU-7, frente a un blanco compuesto por agua destilada. La actividad enzimática, expresada en µmol de H_2O_2 min^{-1} ml^{-1} se calculó a partir de la velocidad inicial de la reacción y de un coeficiente de extinción molar para el H_2O_2 de 39,58 M^{-1} cm^{-1} (del Río *et al.*, 1977).

2.2.4. Actividad ascorbato peroxidasa (APX; EC 1.11.1.11)

Se siguió el método descrito por Jiménez *et al.* (1997), basado en la medida espectrofotométrica a 290 nm de la oxidación del ascorbato en presencia de H_2O_2. La reacción se llevó a cabo a 25 ºC en un volumen de reacción de 1 ml formado por tampón Hepes-NaOH 50 mM, pH 7.6, con ascorbato 0.2 mM, 100 µl de muestra y H_2O_2 0.3 mM.

La reacción se inició añadiendo H_2O_2 y se siguió el descenso de la absorbancia con un espectrofotómetro Beckman modelo DU-7, durante 3 min. Se registraron también blancos de muestra, conteniendo éstos solo muestra y tampón con ascorbato. La actividad enzimática, expresada en nmol de ácido ascórbico oxidado min^{-1} ml^{-1}, se calculó aplicando un coeficiente de extinción molar (ε_{290}) para el ácido ascórbico de 2.8 mM^{-1} cm^{-1} (Hossain y Asada, 1984).

2.4.5. Actividad glutation reductasa (GR; EC 1.6.4.2)

Se siguió el método de Carlberg y Mannervik (1985), que estima la actividad GR a través de la medida de la tasa de oxidación del NADPH necesaria para transformar el glutation oxidado (GSSG) en glutation reducido (GSH). La reacción se llevó a cabo a 25 ºC en un volumen de reacción de 1 ml con la siguiente composición: tampón HEPES-Na 0.1 M, pH 7.8, con EDTA 1 mM, $MgCl_2$ 3 mM y GSSG 0.5 mM; extracto enzimático (150 µl); y NADPH 0.2 mM, preparado en el mismo tampón de reacción aunque sin GSSG. La reacción se siguió durante un tiempo de 2 min midiéndose la caída de la absorbancia del NADPH a 340 nm con un espectrofotómetro Beckman modelo DU-7.

Además de las reacciones problema, se realizaron sendas reacciones control, de muestra y de reactivos, para corregir de este modo la oxidación del NADPH no atribuible a la GR. La composición de estos blancos fue la siguiente:

	Blanco de reactivos	Blanco de muestra
Tampón sin GSSG	+	+
Tampón con GSSG	+	-
NADPH	+	+
Muestra	-	+

La actividad enzimática, expresada en nmol de NADPH oxidado min^{-1} ml^{-1}, se calculó a partir de la máxima pendiente obtenida y aplicando un coeficiente de extinción molar para el NADPH a 340 nm (ε_{340}) de 6.22 mM^{-1} cm^{-1} (Jiménez *et al.*, 1997).

3. DETERMINACIONES ANALÍTICAS

3.1. Daño oxidativo a lípidos

Se siguió el método descrito por Minotti y Aust (1987), basado en la cuantificación espectrofotométrica del complejo formado entre el ácido tiobarbitúrico (TBA) y el malondialdehído (MDA) que resulta de la degradación de los ácidos grasos poliinsaturados a causa de su peroxidación. Para el ensayo, se adicionaron 200 µl de muestra a 1 ml de mezcla de reacción con la siguiente composición:

Ácido tricloroacético al 15% (p/v)
Ácido tiobarbitúrico al 0.375% (p/v)
Butilhidroxitolueno (BHT) al 0.01% (p/v)
HCl 0.25 N

Los reactivos se prepararon en H_2O Milli-Q y el ensayo se llevó a cabo en tubos escrupulosamente limpios. La mezcla de la muestra con la solución reactiva se calentó a 100 °C durante 15 min. Una vez fría, la muestra se centrifugó a 800 g durante 5 min en una microcentrífuga y se midió la absorbancia del sobrenadante a 535 nm. Para determinar el contenido de MDA de las muestras en nmol MDA g^{-1} pf, se preparó una curva patrón de MDA a partir de una solución madre de malondialdehído bis-dimetilacetal 100 µM en agua ultrapura con las siguientes concentraciones 0.1, 0.25, 1, 5 y 10 µM. La concentración en MDA de las muestras problema se calculó interpolando los valores de absorbancia obtenidos a 535 nm en la recta de regresión lineal obtenida con la curva patrón de MDA.

3.2. Prolina y azúcares solubles totales

La prolina y los azúcares totales fueron obtenidos a partir de 1 g de peso fresco siguiendo el siguiente procedimiento descrito por Bligh y Dyer (1959):

1. Homogeneizar con vórtex la muestra 1 min con 3,75 ml de metanol.
2. Repetir durante 1 min con 3,75 ml de metanol.
3. Homogeneizar con vórtex durante 1 min con 7,5 ml de cloroformo.

4. Homogeneizar con 3,75 ml de una solución de H_2O salada al 0.88% (p/v)
5. Centrifugar a 5000 rpm durante 10 min a 0°C en una centrífuga refrigerada.
6. Usar el extracto metanólico (superior) para la determinación de azúcares, prolina, ácidos orgánicos...

Para la determinación de la prolina se siguió el método descrito por Bates *et al.*, (1973) basado en la cuantificación a 530 nm de la prolina extraída con tolueno.

Se hace reaccionar 1 ml de muestra obtenida como se ha mencionado anteriormente con 1 ml de reactivo de ninhidrina y 1 ml de ácido acético glacial. Durante 1 hora se lleva la mezcla a ebullición hasta que la reacción se detiene metiendo los tubos en hielo picado. Se extrae la prolina con 2 ml tolueno agitando en un vórtex 15-20 seg. Medir absorbancia a 530 nm usando tolueno como blanco. Para cuantificar la prolina y expresarla como nmol prolina g^{-1} pf, se realizó una curva patrón a partir de una solución madre de prolina 1 mM con las siguientes concentraciones 0, 25, 50, 100, 200 y 300 μM de prolina.

Reactivo de ninhidrina: Calentar 1,25 g de ninhidrina en 30 ml ácido acético glacial y 20 ml de ácido fosfórico 6M agitando hasta disolver. Tener mucha precaución con los vapores tóxicos. Se conserva a 4°C menos de 24 h.

La determinación de los azúcares totales se llevó a cabo según el método propuesto por Irigoyen *et al.* (1992). Se hicieron reaccionar 100 μl de la muestra obtenida como se ha mencionado anteriormente con 3 ml de reactivo de antrona. Se hierve 10 min a 100 °C. Dejar enfriar y medir absorbancia a 620 nm en un espectrofotómetro Shimadzu UV-1603 (Shimadzu, Tokio, Japón) usando como curva patrón glucosa a unas concentraciones 20-400 μg/ml y como blanco agua destilada.
El resultado se expresa como mg TSS · g^{-1} pf

Reactivo de antrona: 200 mg antrona + 100 ml H_2SO_4 al 72% (v/v). Es un reactivo extemporáneo. Tener mucha precaución con los vapores tóxicos.

3.3. Peróxido de hidrógeno

Se siguió el método de Frew *et al.* (1983), que se basa en la cuantificación del H_2O_2 a través de la oxidación de la 4-aminoantipirina catalizada por una peroxidasa. El ensayo se llevó a cabo en un volumen de reacción de 10 ml compuesto por 4 ml de solución reactiva, 50-200 μl de muestra y agua Milli-Q hasta completar un volumen de 10 ml. Asimismo, el ensayo se llevó a cabo sobre una curva patrón de H_2O_2 con concentraciones 0, 1, 2, 3, 4, 5, 10, 15 y 20 μM para poder interpolar en ella los resultados de la absorbancia de las muestras.

La solución reactiva estaba compuesta por fenol 0,234 % (p/v), 4-aminoantipirina 0,10 % (p/v), peroxidasa de rábano 20 nM, H_2O_2 2,5 μM y tampón fosfato-K 0,1 M, pH 6.9. Esta solución se incubó con las muestras (o con las distintas concentraciones de H_2O_2 de la curva patrón) a temperatura ambiente durante 5 min, y a continuación se

midió la absorbancia a 505 nm frente a un blanco formado por 4 ml de la solución reactiva y 6 ml de agua Milli-Q.

El resultado finalmente se expresa en nmol H_2O_2 g^{-1} pf.

3.4. Fijación de nitrógeno

3.4.1. Actividad reductasa del acetileno (ARA)

La actividad nitrogenasa se estimó por la técnica de reducción del acetileno (Hardy *et al.*, 1973). Aunque ARA medido en botes cerrados no representa la verdadera actividad nitrogenasa (Minchin *et al.*, 1983), sí que puede ser apropiada en ensayos para comparar resultados entre diferentes tratamientos (Irigoyen *et al.*, 1992).

El procedimiento que se siguió fue el siguiente:

Las raíces con los nódulos intactos se metieron en frascos de vidrio herméticamente cerrados de 300 ml y se añadió 15 ml de C_2H_2. El frasco fue incubado a temperatura ambiente durante 15 min. Posteriormente, se tomaron muestras de 500 µl del frasco y se cuantificó el contenido de etileno con un cromatógrafo de gases Hewlett Packard modelo 5890 equipado con una columna Poropak-R y un detector de llama de hidrógeno.

4. DETERMINACIONES FISIOLÓGICAS

4.1. Producción de biomasa

En el momento de la cosecha, el sistema radical se separó del suelo y se determinó el peso fresco de los nódulos cuando se trabajó con soja. Se determinó igualmente el peso fresco de raíz y parte aérea. El peso seco de la parte aérea se midió después de secarlo en un horno a 70 °C durante 2 días.

4.2. Potencial hídrico

El potencial hídrico (Ψ) al mediodía se determinó mediante una cámara sicrométrica y un microvoltímetro HR-33T (Wescor Inc, Logan, UT, USA) siguiendo el método "dew point". Para ello, se cortaron discos de hoja correspondientes a la tercera hoja más joven, se colocaron en la cámara C52 y se dejó que alcanzaran un equilibrio de temperatura y vapor de agua durante 15 min antes de las medidas.

4.3. Contenido relativo de agua

El contenido hídrico relativo (CHR) en parte aérea de planta se determinó en el momento de la cosecha siguiendo el método de Barrs y Weatherley (1962). Se pesan (peso fresco, PF) cinco discos de hoja (1 cm diámetro) de cinco hojas por planta inmediatamente después de cosechar la planta. Posteriormente se depositan en una placa de Petri con agua destilada durante 3 h a 25 °C y bajo una luz fluorescente de 40 W, y se determinan los pesos turgentes (PT). Luego se secan las muestras en un horno a 60 °C durante 24 h para obtener los pesos secos (PS). El contenido relativo de agua se calcula como:

$$CHR = \frac{PF - PS}{PT - PS}$$

4.4. Intercambio gaseoso

La tasa de intercambio de CO_2, la tasa de transpiración , la eficiencia del uso de agua (WUE) y la conductancia estomática se midieron en la tercera hoja de cada planta.

El CO_2 atmosférico se tomó a 5 metros del suelo. La densidad de flujo fotosintético de fotones (PPFD) fue 1180 μmol m^{-2} s^{-1} para asegurar que no tenía lugar limitación en la intensidad luminosa. La luz la aportó una lámpara halógena (General Electric 300 PAR 56/WFL). Se usó un modelo portátil LCA-3 de un analizador de CO_2 (Analytical Development Co., Hoddesdon, UK). Las medidas se hicieron 2 h después de iniciarse el fotoperíodo diurno.

5. TÉCNICAS DE BIOLOGÍA MOLECULAR

5.1. Plásmido

Se utilizó el plásmido pGEM-T Easy (Promega, USA). Es un vector de clonación en *E. coli* con colas de adenina típicas de PCR resultantes de la actividad transferasa terminal de la Taq polimerasa sin actividad correctora de prueba. El sitio de clonación se encuenra en el marco del gen LacZ, lo que permite la selección de los plásmidos portadores del inserto mediante un escrutinio de colonias blancas/azules cuando se cultivan en presencia de X-gal. La selección se realiza por la tolerancia al antibiótico ampicilina que confiere este plásmido.

5.2. Extracción de ADN

La extracción de ADN genómico de diferentes plantas y hongos MA se llevó a cabo empleando el kit Dneasy Plant Mini kit de Quiagen (USA), siguiendo las instrucciones del fabricante.

5.3. Cuantificación del ADN

El ADN extraído se cuantificó con la ayuda de un espectrofotómetro. Para ello se utilizaron cubetas de cuarzo. La absorbancia de una alícuota de 5 μl de muestra diluida en 995 μl de agua milli-Q estéril se midió a 230, 260 y 280 nm. Cada unidad de absorbancia a 260 nm se consideró como 50 μg/ml de ADN. Para poder conocer una posible contaminación por carbohidratos se calculó la relación entre las absorbancias obtenidas a 260 y 230 nm, debiendo ser ésta superior a 2 para considerar una muestra libre de proteínas y/o fenol, la relación entre las absorbancias a 260 y 280 nm debía tomar una valor superior a 1.8.

5.4. Reacción en cadena de la polimerasa (PCR)

En tubos de 0.2 ml se preparó la siguiente mezcla de reacción:

x	ADN molde (10-100 ng)
2.5 μl	Tampón 10x de la Taq DNA polimerasa termoestable
0.75 μl	$MgCl_2$ 50 mM
5 μl	dNTPs 1 mM
0.5 μl	cebador 5' (10 μM)
0.5 μl	cebador 3' (10 μM)
0.5 μl	Taq DNA polimerasa termoestable (5 U/μl)
15.25 - x μl	Agua bidestilada estéril hasta 25 μl

También se preparó un tubo de microcentrífuga sin ADN como control negativo para poder descartar posibles artefactos o contaminaciones. Tras una agitación rápida se centrifugaron y se colocaron en un termociclador (Perkin-Elmer Modelo 2400) y se utilizó un programa específico en función de los cebadores usados y de la diana de ADN a amplificar.

5.5. Visualización del ADN amplificado mediante electroforesis en gel de agarosa

La separación electroforética del ADN se realizó en geles de agarosa al 1.2% en tampón TAE. La electroforesis se llevó a cabo en tampón TAE (1x) a 100 V. Las muestras se prepararon en tampón de carga 1x. La visualización del ADN en los geles se hizo mediante tinción con bromuro de etidio y fotografía del gel expuesto a la luz UV (260 nm).

Tampón TAE (50x): Tris 242 g/l, $Na_2EDTA \cdot 2H_2O$ 37,2 g/l, ácido acético glacial 57,2 ml/l y agua destilada.
Tampón de carga (6x): sacarosa 50% (p/v), azul de bromofenol 0,3%

5.6. Purificación de fragmentos de ADN a partir de geles de agarosa

Para ello se empleó el kit QiaexII Gel Extraction kit de Qiagen (Hilden, Germany) siguiendo las instrucciones del fabricante.

5.7. Ligación en vectores de clonación

La ligación entre un fragmento de ADN y el vector de clonación adecuado se realizó usando una relación molar inserto:vector de 3:1 y teniendo en cuenta que se necesitan 50 ng de vector.
En un tubo de microcentrífuga se incubó durante toda una noche a 4 °C la siguiente mezcla de reacción:

x μl	inserto ADN
1 μl	T_4-ADN Ligasa
1 μl	Vector pGEM-t-Easy (50 ng/μl)
x+2 μl	Tampón de ligación (2x)

Notas: el vector pGEM-t-easy, el tampón y el enzima ligasa han sido suministrados por Promega.

5.8. Transformación de células de *Escherichia coli*

Tiene por objeto introducir plásmidos con el inserto deseado en cepas de *E. coli*. Para transformar las células quimiocompetentes se utilizó la metodología descrita por Rodríguez y Tait (1983), siguiendo el protocolo que se indica a continuación:

1. Enfriar una alícuota de 100 μl de células competentes en hielo hasta su completa descongelación.
2. Añadir a dicha alícuota 4 μl de plásmido (del tubo preparado en el apartado anterior) y agitar suavemente.
3. Incubar en hielo 30 min.
4. Incubar durante 60 seg a 42 ºC y a continuación enfriar en hielo 2 min.
5. Añadir 900 μl de medio LB líquido estéril e incubar 1 h en agitación a 37ºC
6. Se prepararon placas de Peti con medio de cultivo LB sólido suplementado con ampicilina 100 μg/ml. Se extendieron en su superficie 40 μl de IPTG y X-gal a una concentración de 20 mg/ml. Se dejan 20-30 min para asegurarnos de su completa absorción.
7. Sembrar 50 μl, 100 μl y 200 μl de cultivo en placas de medio selectivo que se incuban toda la noche a 37 ºC.
8. Se hacen réplicas de las colonias blancas obtenidas, transformadas con el vector con el inserto, para su posterior estudio.

5.9. Purificación del ADN plasmídico

La purificación del ADN del plásmido se llevó a cabo usando un kit QIAprep Spin Miniprep kit (Qiagen) siguiendo las instrucciones del fabricante.

5.10. Construcción y análisis de genoteca de ADNc

Una vez extraído el ARN del micelio de *Glomus intraradices* crecido en cultivo monoxénico y sometido a déficit hídrico por la adición de 25% PEG 6000, se procedió a sintetizar ADNc usando el kit Smart PCR cDNA Synthesis Kit (Clontech, Palo Alto, California) y se clonó en el bacteriófago lambda ExCell (Amersham, Little Chalfont, UK). Se hizo un escrutinio de la genoteca mediante hibridación diferencial de colonias idénticas. Se siembran en placa aproximadamente 10^4 partículas de fago λ a baja densidad (1500-2000 unidades formadoras de placa por placa de petri) en la bacteria hospedadora *E. coli* (cepa NM522). Tras la incubación durante 7-8 h a 37 ºC, aparecieron las colonias y se guardaron las placas a 4 ºC durante 1 h para solidificar la agarosa. Posteriormente se transfirieron a una membrana de nylon Hybond-N+ (Amersham, Little Chalfont, UK) y se dejaron 2 min a temperatura ambiente (para el primer filtro) o durante 4 min (para el segundo filtro). El ADN se desnaturalizó y se fijó autoclavando el filtro durante 5 min a 120 ºC. Cada uno de los filtros se hibridó a 65 ºC con una sonda de ADNc total radiactiva de micelio de *G. intraradices* crecido *in vitro* en

presencia o ausencia de 25% PEG 6000. Tras lavar dos veces durante 5 min a temperatura ambiente con 2x SSC y 0.1% SDS y otras dos veces durante 15 min a 65 °C con 0.5x SSC y 0.1% SDS, las membranas se expusieron toda la noche sobre películas Kodak X-RAY-OMAT a –70 °C.

Los clones detectados de manera diferencial se usaron como moldes para un escrutinio basado en la PCR con cebadores universales M13 para verificar la pureza de los clones y el tamaño de los insertos. Los productos de PCR obtenidos se dividieron en dos fracciones iguales, se separaron en geles de agarosa y se transfirieron a membranas de nylon (Martin-Laurent *et al.*, 1995). Se hizo un análisis southern blot para confirmar los clones expresados diferencialmente hibridando las membranas con ADNc marcado con ^{32}P de micelio de *G. intraradices* crecido *in vitro* en presencia o ausencia de 25% PEG 6000.

5.11. Extracción de ARN

5.11.1. Extracción de ARN de raíces y hojas

La extracción de ARN se hizo tanto en raíz como en hoja partiendo de 1 g de peso fresco congelado a –80 °C

El procedimiento fue el siguiente:

1. Homogeneizamos en un mortero con nitrógeno líquido el material de partida.
2. Añadir 1800 µl de tampón REB y seguir homogeneizando.
3. Dejar descongelar y añadir rápidamente 1800 µl de fenol:cloroformo:isoamílico en proporción 50:24:1
4. Traspasar el material a tubos estériles de 2 ml, agitar varias veces de forma no agresiva. Mantener en hielo
5. Centrifugar 10 min a 12 000 rmp a 4°C. Recoger la fase acuosa y reextraer dos veces con 900 µl de fenol:cloroformo:isoamílico en proporción 25:24:1
6. Recoger la fase acuosa y pasarla a un nuevo tubo. Medir el volumen añadido y adicionar 1/3 del volumen de la muestra de ClLi 8M, poco a poco en el vórtex a baja velocidad para ir adquiriendo la concentración final de 2 M.
7. Incubar en hielo durante toda una noche.
8. Centrifugar a máxima velocidad en una microcentrífuga durante 30 min a 4°C.
9. Eliminar el sobrenadante y lavar el precipitado con 300 µl de LiCl 2M frío. Centrifugar de nuevo durante 10 min en las mismas condiciones.
10. Recoger el precipitado en 200 µl de tampón TE y precipitar de nuevo con 2.5 volúmenes de etanol 100% y 0,1 volumen de acetato sódico 3M pH 5.2.
11. Incubar toda una noche a –20 °C
12. Recoger por centrifugación a máxima velocidad durante 30 min y 4 °C
13. Lavar con 300 µl de etanol 75% y volver a centrifugar 5 min.
14. Secar el tubo con mucho cuidado y resuspender el precipitado en 50-60 µl de H_2O-DEPC

H_2O-DEPC: Añadir 1 ml de dietil-pirocarbonato por cada litro de agua destilada. Agitar durante toda una noche. Se autoclava y se agita con el tapón abierto dentro de una campana extractora para eliminar gases tóxicos.
Tampón REB: Tris-HCl 25 mM, EDTA 25 mM, NaCl 75 mM, SDS 1%, β-mercaptoetanol 1M. Preparar en H_2O-DEPC.
Tampón TE: Tris 10 mM pH 8, EDTA 1 mM. Preparar en H_2O-DEPC.
Notas: Usar guantes durante todo el proceso para evitar el contacto del ARN con RNasas que puedan degradarlo. Todo el material utilizado ha de estar completamente estéril. Los reactivos tienen que estar fríos. Autoclavar todos los reactivos salvo el tampón REB que se autoclava antes de añadir β-mercaptoetanol.

5.11.2. Extracción de ARN de nódulos

La extracción de ARN de nódulos de soja se hizo partiendo de 0.3 g de peso fresco de nódulos, que habían sido previamente separados de las raíces en el momento de la cosecha y almacenados a –80 °C para su posterior uso.

Se utilizó el RNeasy Plant Mini Kit (Quiagen, Hilden, Germany), siguiendo las instrucciones del fabricante.

5.12. Cuantificación del ARN

La cuantificación del ARN se realizó espectrofotométricamente siguiendo la metodología descrita por Sambrook *et al.* (1989). Para ello se utilizaron cubetas de cuarzo. La absorbancia de una alícuota de 5 µl de muestra diluida en 995 µl de H_2O-DEPC fue medida a 230, 260 y 280 nm. Cada unidad de absorbancia a 260 nm fue considerada como 40 µg/ml de ARN. Para poder conocer una posible contaminación por carbohidratos se calculó la relación entre las absorbancias obtenidas a 260 y 230 nm, debiendo ser ésta superior a 2. para considerar una muestra libre de proteínas y/o fenol la relación entre las absorbancias a 260 y 280 nm debía ser superior a 1,8.

5.13. Tratamiento del ARN a la DNasa

En un tubo de microcentrífuga se añaden los siguientes componentes:

ARN	30 µg
Inhibidor de la Rnasa (40 U/µl)	1 µl
Tampón 10x	6 µl
DNasa (10 U/µl)	2 µl
Agua-DEPC	hasta volumen final de 60 µl
	60 µl

1. La mezcla se incuba a 37 °C durante 30 min
2. Añadir el mismo volumen de H_2O-DEPC (60 µl)
3. Extraer con 2 volúmenes de fenol:cloroformo:isoamílico en proporción 25:24:1. Centrifugar a 13.400 g y 4 °C durante 10 min.
4. Precipitar el sobrenadante con 0,1 volumen de AcNa 3M pH 5.2 y 2.5V de etanol 100%
5. Incubar 2 h a –20 °C

6. Centrifugar a 13.400 g, 4 °C durante 30 min
7. Lavar el precipitado con etanol 75%
8. Secar cuidadosamente el tubo y resuspender el precipitado en 21 µl de H_2O-DEPC. Conservar el ARN a –80 °C

5.14. Northern blot

5.14.1. Electroforesis del ARN en gel de agarosa

Para determinar la calidad del ARN extraído se preparó un gel de agarosa 1,2% en condiciones desnaturalizantes. Se tomaron 15 µg de ARN y se le añadió 10 µl de tampón de muestra y 1 µl de tampón de carga con bromuro de etidio. A continuación, se desnaturalizó el ARN incubando esta mezcla a 65 °C durante 15 min y las muestras se introdujeron inmediatamente en hielo antes de cargar el gel. La electroforesis se desarrolló en un tampón MOPS 1x a un voltaje constante de 80 V. Para visualizar el ARN se expuso el gel a luz ultravioleta.

Tampón de electroforesis MOPS (10x): MOPS 200 mM, ajustar el pH a 7 con NaOH. Añadir posteriormente acetato sódico 50 mM y EDTA 10 mM. Preparar todos los reactivos en H_2O-DEPC y autoclavar a 120 °C durante 20 min.
Tampón de muestra (1x): 100 µl formamida desionizada, 20 µl tampón de electroforesis MOPS 10x, 38 µl formaldehído, 42 µl H_2O-DEPC. Esta solución se conserva a –20 °C hasta 6 meses.
Tampón de carga (10x): 50% sacarosa, 0,3% azul de bromofenol, 1 µg/ml bromuro de etidio. Esta solución se prepara en tampón MOPS 1x.
Gel de agarosa en condiciones desnaturalizantes: Para un volumen de 50 ml: En el microondas se calienta 0,6 g de agarosa, 5 ml de tampón de electroforesis MOPS 10x y 42,5 ml de H_2O-DEPC. Tras la fusión se controla la temperatura y cuando ésta baja de 60°C se le adiciona 2,5 ml de formaldehído al 37%. Todo este proceso ha de realizarse en campana extractora.
Notas: las cubetas de electroforesis han de limpiarse antes de su uso con solución desnaturalizante compuesta por NaOH 0,1M, EDTA 20 mM durante 15 min. Después se neutraliza con HCl 0,1M durante 5 min y por último se lavan varias veces con agua milli-Q.

5.14.2. Transferencia del ARN a membrana

La transferencia de los ARNs se realizó a una membrana de nylon cargada positivamente. Dicha membrana se cortó del tamaño del gel junto con dos trozos de papel absorbente tipo Whatman nº 3 y se incubaron en agitación y temperatura ambiente durante 15 min en SSC 2x. El gel se incubó en agitación y temperatura ambiente en solución SSC 20x durante 25 min.

La transferencia se dejó toda la noche. Posteriormente la membrana se retira y para fijar el ARN a la membrana, se somete a luz UV durante 3 min y 45 seg.

SSC 20x: NaCl 3M, citrato sódico 0,3M. Ajustar a pH 7 con NaOH 10N. Autoclavar a 120 °C durante 20 min.

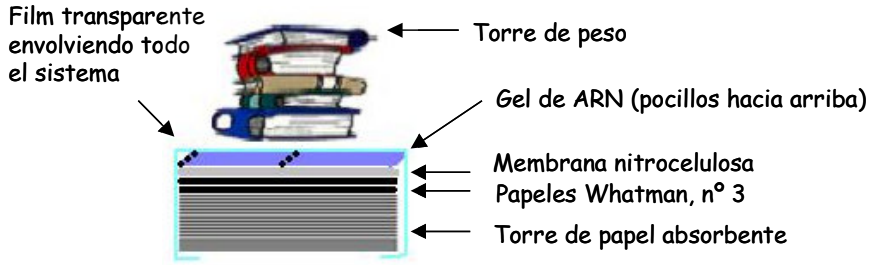


Figura 2. Esquema representativo de la disposición de elementos durante la transferencia del ARN a membrana. Sobre una torre de papel absorbente se colocan 2 trozos del mismo tamaño del gel de papel Whatman, preferiblemente del nº 3. Sobre estos, se dispone la membrana previamente cortada al tamaño del gel, a continuación el gel con los pocillos hacia arriba y encima de todo film transparente que cubra todo el dispositivo perfectamente para evitar pérdidas. Por último se coloca un peso para asegurar la transferencia.

5.14.3. Síntesis de la sonda marcada radiactivamente mediante PCR

La mezcla de reacción fue la siguiente:

2 µl ADN (10 ng de plásmido conteniendo el inserto de ADN a marcar)
2 µl Tampón de la Taq polimerasa (10x)
1 µl $MgCl_2$ 50 mM
1 µl dATP (100 µM)
1 µl dGTP (100 µM)
1 µl dTTP (100 µM)
5 µl ^{32}P -dCTP (10 µcurios/µl)
1 µl Cebador M13 5' (10 µM)
1 µl Cebador M13 3' (10 µM)
0,2 µl Taq Polimerasa
4.8 µl H_2O destilada estéril

El programa de PCR seguido fue el que se muestra a continuación:
(95 °C, 5 min) x 1 ciclo
(94 °C, 45 seg; 56 °C, 30 seg; 72 °C, 45 seg) x 32 ciclos
(72 °C, 5 min; 4 °C, ∝) x 1 ciclo

5.14.4. Purificación de las sondas marcadas radiactivamente

Para purificar las sondas marcadas se utilizó el kit comercial Mini Quick Spin TM Columns, (Boehringer Manheim, Indianapolis, IN, USA) que retiene y purifica el ADN marcado.

5.14.5. Pre-hibridación e hibridación

El procedimiento que se siguió fue el siguiente:

1. La membrana se coloca en un tubo de hibridación con el ARN hacia arriba y pre-hibridó durante al menos 2 h con la solución de hibridación a 65 °C y agitación.

2. Paralelamente la sonda se desnaturalizó calentando a 95 °C durante 5 min e incubando inmediatamente en hielo 5 min.
3. La solución de hibridación se elimina del tubo y se añade nueva solución de hibridación.
4. La membrana hibrida toda la noche a 65 °C en agitación.
5. La solución de hibridación se elimina y se llevan a cabo los siguientes lavados:

- 2 lavados de 5 min con 2x SSC + 0.1% SDS a temperatura ambiente.
- 2 lavados de 15 min con 0.5x SSC + 0.1% SDS a 65 °C y agitación.

Solución de hibridación: Denhardt (5x), SSC (5x), SDS 0,5%

5.14.6. Detección y revelado

Las membranas se expusieron a películas fotográficas KODAK X-RAY-OMAT a –80 °C, protegidas con cassettes especiales. El tiempo de exposición dependió del grado de marcaje que presentaban las membranas tras los lavados.

El revelado se realizó sumergiendo las películas en líquido de revelado fotográfico durante 3 ó 4 min, dependiendo de la señal obtenida. Tras esto, las películas se fijaron sumergiéndolas en líquido fijador fotográfico durante otros 3 ó 4 min. Las películas se lavaron con agua para eliminar restos del líquido fijador y se dejaron secar al aire. Las bandas obtenidas se cuantificaron utilizando el programa informático *Quantity One* (Bio-Rad- Hemel Hempstead, UK).

5.15. Análisis de la expresión génica mediante técnicas de PCR cuantitativa a tiempo real

5.15.1. Retrotranscripción *in vitro*: síntesis de ADNc

La síntesis del ADNc se realizó a partir de 1 μg de ARN total usando la reversotranscriptasa SuperScript II (Invitrogen, USA) por el método de los cebadores aleatorios, usando las condiciones del fabricante, que se detallan a continuación:

1. Se mezclan en un tubo de microcentrífuga:

   1 μg ARN total
   1 μl Cebadores aleatorios (100 ng/μl, Boehringer, Alemania)
   1 μl dNTP mix (10 mM)
   hasta 12 μl H_2O-DEPC
2. Mezclar por pipeteo e incubar la mezcla 5 min a 65 °C
3. Pasar a hielo inmediatamente y añadir:
   4 μl Tampón de primera cadena
   2 μl 0,1 M DTT
   1 μl Inhibidor de RNasa, (Invitrogen, 40U/μl)
   consiguiendo un volumen total de 19 μl.
4. Mezclar suavemente e incubar 2 min a 25 °C

5. Añadir 1 µl del enzima SuperScript II RT (200 U/µl) y mezclar pipeteando suavemente.
6. Incubar 50 min a 42 °C.
7. Inactivar la reacción incubando 15 min a 70 °C
8. El tubo de microcentrífuga con el ADNc se guardó a –20 °C

5.15.2. PCR cuantitativa a tiempo real

Las PCRs cuantitativas a tiempo real se realizaron en un termociclador iCycler (BioRad).

Cada reacción contenía:

1 µl	dilución 1:10 ADNc
2,5 µl	Tampón 10x sin Mg
1,5 µl	$MgCl_2$ 50 mM
5 µl	dNTPs 10 mM
0,5 µl	cebador 5' (10 µM)
0,5 µl	cebador 3' (10 µM)
2,5 µl	solución SyBr Green 1x en agua Milli-Q
0,1 µl	Taq Platinum (Invitrogen, 5 U/µl)

Completar con agua hasta alcanzar los 25 µl

Solución de SyBr Green (500x):18µl DMSO, 1 µl fluoresceína (Molecular Probes), 1 µl SyBr Green (Molecular Probes). Proteger de la luz, guardar a 4 °C y desechar tras 7-10 días.

Se utilizó en cada caso un programa de PCR específico en función de los cebadores utilizados y de la diana de ADN a amplificar.

5.16. Secuenciación

Las reacciones de secuenciación se realizaron en el Servicio de Secuenciación del Instituto de Parasitología y Biomedicina López Neyra (CSIC, Granada). Las secuencias se determinaron mediante secuenciación de cadena única, usando un secuenciador automático Perkin-Elmer ABI Prism 373. Cada reacción consistía de 100-200 ng de ADN molde y 6.4 pmol de cebador.

6. MÉTODOS BIOINFORMÁTICOS

6.1. Identificación de la secuencia de interés en las bases de datos

Con el fin de encontrar genes implicados en la tolerancia al déficit hídrico, se analizaron las bases de datos públicas disponibles en la red (http://www.ebi.ac.uk). Las secuencias allí encontradas se compararon con las secuencias aminoacídicas de genes de otros organismos disponibles en las bases de datos usando el algoritmo Blastx (Altschul *et al.*, 1990). Los alineamientos múltiples se llevaron a cabo con el programa CLUSTALW (Thompson *et al.*, 1994).

6.2. Diseño de cebadores

Los cebadores se diseñaron empleando el programa Amplify 3.1.4 para MacOS, Bill Engels, University of Wisconsin (USA) . Para más información visitar la página web http://engels.genetics.wisc.edu/amplify/index.html.

6.3. Comparación de secuencias

El grado de homología de las secuencias obtenidas en este estudio con las de otros organismos presentes en las bases de datos se determinó usando los programas BLAST y FASTA, disponibles en la red (http://www.ebi.ac.uk/o bien en http://www.ncbi.nlm.nih.gov/).

6.4. Obtención de secuencia aminoacídica

La determinación de la secuencia aminoacídica a partir de la secuencia nucleotídica se realizó con la herramienta "Translate" de Expasy (http://expasy.org).

7. ANÁLISIS ESTADÍSTICO

Los resultados de todas las gráficas y tablas presentadas en esta Tesis Doctoral se sometieron a un ANOVA. Cuando se detectaron diferencias significativas como consecuencia de los tratamientos aplicados, se procedió a calcular estas mediante el test de rango múltiple de Duncan (Duncan, 1955) a un nivel de significación $P< 0.05$.

En los experimentos de regulación de la expresión génica, la cuantificación de la expresión se llevó a cabo mediante PCR cuantitativa a tiempo real. Los experimentos se repitieron tres veces y el valor del Ct de cada muestra se determinó por triplicado. Se seleccionó un experimento representativo y se calculó la media de las tres repeticiones y el error estándar de la misma.

ACTIVIDADES ANTIOXIDANTES EN PLANTAS MICORRIZADAS DE SOJA BAJO DÉFICIT HÍDRICO Y SU POSIBLE VINCULACIÓN CON EL PROCESO DE SENESCENCIA NODULAR

Resumen

Los mecanismos por los cuales la simbiosis micorrícica protege a las plantas de soja (*Glycine max*) frente a la senescencia nodular prematura inducida por déficit hídrico se investigan aquí mediante la evaluación de la actividad de un conjunto de enzimas antioxidantes y su relación con la senescencia nodular. Se procedió a determinar las actividades superóxido dismutasa (SOD), catalasa (CAT), ascorbato peroxidasa (APX) y glutation reductasa (GR) en plantas de soja en condiciones óptimas y sometidas a déficit hídrico, inoculadas con *Bradyrrhizobium japonicum* solamente o en combinación con *Glomus mosseae.* En raíz, solo la actividad GR fue mayor en las plantas micorrizadas que en las no micorrizadas. Las otras actividades antioxidantes fueron similares, o más bajas (APX) en plantas micorrizadas sometidas a estrés que en las correspondientes plantas no micorrizadas. Igualmente, en nódulos, las actividades SOD, CAT y APX fueron menores en plantas micorrizadas sometidas a estrés hídrico que en plantas no micorrizadas, mientras que, de nuevo, la actividad GR fue mayor en nódulos de plantas micorrizadas. Por lo tanto, proponemos que la existencia de una actividad GR consistentemente mayor en raíces y nódulos de plantas micorrizadas puede haber contribuido a disminuir el daño oxidativo a biomoléculas que están implicadas en la senescencia nodular prematura. Mecanismos adicionales de evitación de la sequía inducidos por la simbiosis MA pueden también haber contribuido a un menor estrés oxidativo en plantas micorrizadas.

Palabras clave: antioxidante, simbiosis micorrícico arbuscular, sequía, senescencia nodular.

ANTIOXIDANT ACTIVITIES IN MYCORRHIZAL SOYBEAN PLANTS UNDER DROUGHT STRESS AND THEIR POSSIBLE RELATIONSHIP TO THE PROCESS OF NODULE SENESCENCE

Summary

The mechanisms by which the mycorrhizal symbiosis protects soybean (*Glycine max*) plants against premature nodule senescence induced by drought stress is investigated here by evaluating the activity of a set of antioxidant enzymes in relation to nodule senescence. The activitiy of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) was determined in well-watered or drought-stressed soybean plants inoculated with *Bradyrrhizobium japonicum* alone or in combination with *Glomus mosseae*. In roots, only the GR activity was higher in mycorrhizal than in non-mycorrhizal plants. The other antioxidant activities were similar (SOD and CAT) or lower (APX) in droughted mycorrhizal plants than in the corresponding non-mycorrhizal ones. Similarly, in nodules, the SOD, CAT and APX activities were lower in droughted mycorrhizal plants than in non-mycorrhizal plants while, again, the GR activity was higher in nodules from mycorrhizal plants. We propose that the consistently higher GR activity in roots and nodules of mycorrhizal plants might have contributed to decrease the oxidative damage to biomolecules, which is involved in premature nodule senescence. The possibility that additional drought-avoidance mechanisms induced by the AM symbiosis also contributed to the lower oxidative stress in mycorrhizal plants is also considered.

Key words: antioxidant, arbuscular mycorrhizal symbiosis, drought, nodule senescence

1.1. Introduction

The role of the arbuscular mycorrhizal (AM) symbiosis in the alleviation of drought-induced nodule senescence in soybean plants has been recently investigated (Ruiz-Lozano *et al.,* 2001). We observed that, under drought conditions, inoculation of soybean plants with the AM fungus *Glomus mosseae* enhanced nodule dry weight and increased its leghemoglobin and protein contents as well as the nodule activity (measured as acetylene reductase activity, ARA). Thus, we demonstrated that AM symbiosis alleviates drought-induced nodule senescence in legume plants.

The process of nodule senescence has been correlated with a marked decline in the major activities involved in removal of reactive oxygen species (ROS) (Evans *et al.,* 1999; Becana *et al.,* 2000). This is important because oxidative damage to biomolecules has been proposed as one of the most important mechanisms triggering nodule senescence in stressed nodules (Gogorcena *et al.,* 1995, 1997; Escuredo *et al.,* 1996; Becana *et al.*, 2000). Legume nodules have a high capacity to produce ROS, even though the concentration of free O_2 in the central zone is only 5-60 nM (Hunt and Layzell, 1993). The high concentration of oxygen-labile proteins, leghemoglobin and catalytic Fe in the nodules and the tendency of the oxygenated form of leghemoglobin to autoxidize are conductive to the production of ROS in the nodule cytosol (Dalton, 1995). This, in turn, can damage biomolecules such as lipids and proteins, thereby contributing to nodule senescence (Escuredo *et al.,* 1996).

The most remarkable result found in our previous study concerned the measurement of oxidative damage to biomolecules. Drought considerably enhanced oxidative damage to lipids and proteins in nodules of non-mycorrhizal plants whereas mycorrhizal treatments were protected against oxidative damage. Therefore, we concluded that the alleviation of oxidative damage in nodules of AM plants could be an important mechanism involved in the protective effects of the AM symbiosis against premature nodule senescence (Ruiz-Lozano *et al.,* 2001).

At this stage of the research two possibilities can be envisaged to explain the low oxidative damage found in nodules of mycorrhizal plants. Either mycorrhizal plants suffered less drought stress due to a primary drought-avoidance effect by the symbiosis (e.g. by direct water uptake by fungal hyphae from sources inaccessible to non-mycorrhizal plants and transfer to the host plant) and that kept plants protected against the generation of ROS, or mycorrhizal plants increased the activities of a set of enzymes involved in the elimination of active oxygen species. Plant cells contain an array of protective and repair systems that minimize the occurrence of oxidative damage. According to Smirnoff (1993), these can be divided into two categories: systems that react with active forms of oxygen and keep them at a low level [i.e. superoxide dismutases (SODs), catalase (CAT), or peroxidases], and systems that regenerate oxidized antioxidants [glutathione (GSH), glutathione reductase (GR), ascorbate and mono- and dehydroascorbate reductases]. The first group of enzymes are involved in the detoxification of $O_2^{\cdot -}$ radicals and H_2O_2, thereby preventing the formation of $^{\cdot}OH$ radicals. The GR, as well as the GSH, are important components of the ascorbate-glutathione pathway responsible for the removal of H_2O_2 in different cellular compartments (Dalton, 1995; Jiménez *et al.,* 1997).

In this study we analyzed the activity of representatives of the two enzymatic categories proposed by Smirnoff (1993) in root and nodule tissues. The aim was to get some clues on the mechanisms by which the mycorrhizal symbiosis protects legume plants against the premature nodule senescence induced by drought stress.

1.2. Materials and Methods

1.2.1. Experimental design and statistical analysis

The experiment consisted of a randomized complete block design with two inoculant treatments: (1) plants inoculated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, strain USDA 110 (Br), (2) plants inoculated with the mycorrhizal fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *B. japonicum* (Gm+Br). Twelve replicates of each treatment were done totalling 24 pots (one plant per pot) so that half of them were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 10 days 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.

1.2.2. 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 days). 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_2O_2 solution for 8 min, then washed several times with sterile water to remove any trace of chemical that could interfere in 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 the sterilized soil/sand mixture. A suspension (2 ml $seed^{-1}$) of the diazotrophic bacterium *Bradyrhizobium japonicum*, strain USDA 110 (10^9 cell ml^{-1}), was sprinkled over the seedling at the time of planting.

Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted of soil, spores, mycelia and infected root fragments. The AM species was *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, isolate BEG 122. Ten grams of inoculum were added to Gm+Br pots at sowing time just below soybean seedlings.

1.2.3. 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 460-500 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.

Volumetric soil water content is the ratio between the volume of water present and the total volume of the soil sample. It is a dimensionless parameter, expressed either as a percentage (% vol) or as a ratio ($m^3 \cdot m^{-3}$). Water was supplied daily to maintain constant soil water content close to field capacity (17% volumetric soil moisture) during the first 5 weeks of plant growth. At this time half of the plants were allowed to dry until soil water content reached 80% field capacity (12% volumetric soil moisture) and maintained under such conditions for 10 days. In order to control the level of water stress, the pot 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 maintain soil water content at the desired level.

Each week throughout the experiment, plants received 10 ml of Hewitt´s nutrient solution lacking N and P (Hewitt, 1952). Three weeks after planting, Br plants received nutrient solution amended with 0.35 mM K_2HPO_4 (Goicoechea *et al.,* 1997). That P concentration was chosen in an attempt to obtain well-watered plants of similar size and P content in both microbial treatments.

1.2.4. Parameters measured

1.2.4.1. Biomass production

At harvest (45 days after planting), the root system was separated from the soil and nodule fresh weight (FW) determined. Shoot dry weight (DW) was measured after drying in a forced draught oven at 70 °C for two days.

1.2.4.2. Symbiotic development

The percentage of mycorrhizal root infection was estimated by visual observation of fungal colonization after clearing washed roots in 18% KOH and staining with 0.05% trypan blue in lactophenol (v/v), according to Phillips and Hayman (1970). Parameters of mycorrhizal colonization were determined according to Trouvelot *et al.* (1986). The colonization frequency (F%) is a ratio between colonized root fragments and total number of root fragments observed. It gives an estimation of the root length colonized by the fungus. The colonization intensity (M%) is an estimation of the amount of root cortex which became mycorrhiza. Finally, the arbuscule abundance a% gives an estimation of the arbuscule richness in the mycorrhizal root fraction. Four replicates per treatment were used.

1.2.4.3. Nodule activity

Nitrogenase activity was estimated by the C_2H_2 reduction technique (Hardy *et al.*, 1973). Although ARA measured in closed vessels does not represent the true nitrogenase activity (Minchin *et al.*, 1983), it can be appropriate in assays for comparative purposes (Irigoyen *et al.*, 1992). Intact nodulated roots were enclosed in a 300-ml glass flask and 15 ml of C_2H_2 were added. The flask was incubated at room temperature for 15 min. Samples of 500 µl were withdrawn from the flask and the ethylene content was quantified with a Hewlett Packard

model 5890 gas chromatograph equipped with a Poropak-R column and a hydrogen flame ionization detector.

1.2.4.4. Hydrogen peroxide concentration and oxidative damage to lipids

For determination of hydrogen peroxide concentration in nodules, aliquots of nodules were homogeneized with an ice-cold potter in HCl 25 mM and filtered through four layers of nylon cloth. The supernatants were adjusted to pH 7.0 for subsequent H_2O_2 quantification, which was performed by the 4-aminoantipyrine method (Frew *et al.*, 1983).

Lipid peroxides from roots and nodules were extracted by grinding 0.5 g or 0.25 g, respectively, with and ice-cold potter and 6 ml of 100 mM potassium phosphate buffer (pH 7.4) as described previously (Ruiz-Lozano *et al.*, 2001). Lipid peroxidation was estimated as the content of 2-thiobarbituric acid-reactive substances (TBARS) and expressed as equivalents of malondialdehyde (MDA) according to Halliwell and Gutteridge (1989).

1.2.4.5. Preparation of root and nodule extracts

Enzymes were extracted at 0-4 °C from 1 g (f wt) root tissues using a mortar and pestle with 50 mg polyvinylpolypyrrolidone (PVPP) and 10 ml of the following optimized medium: 50 mM K-phosphate buffer pH 7.8 containing 0.1 mM EDTA for SOD, CAT and ascorbate peroxidase (APX) (Gogorcena *et al.,* 1995). The same medium supplied with 10 mM β-mercaptoethanol was used for glutathione reductase (Moran *et al.*, 1994). For nodules, enzymes were extracted from 0.3 g (f. wt) aliquots in 5 ml of the above-mentioned media using an ice-cold potter. Extracts were filtered through four layers of nylon cloth and centrifuged at 20,000 g, 20 min, 0-4 ^{0}C. The supernatants were kept at –70 °C for subsequent enzymatic assays.

Soluble protein was determined by the dye binding microassay (Bio-Rad) using BSA as the standard.

1.2.4.6. Enzyme assays

Total SOD activity (EC 1.15.1.1) was measured according to Beyer and Fridovich (1987) based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25 °C. CAT activity (EC 1.16.1.6) was measured by the disappearance of H_2O_2 (Aebi, 1984). The reaction mixture (3ml) contained 10.6 mM H_2O_2. The reaction was initiated by adding 25 μl of the extract and monitoring the change in absorbance at 240 nm and 25 °C for 3 min. APX activity (EC 1.11.1.11) was measured in a 1-ml reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM hydrogen peroxide and 0.5 mM ascorbate. Adding the H_2O_2 started the reaction and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate of ascorbate (Amako *et*

al., 1994). Finally, GR activity (EC 1.6.4.2) was determined by the procedure of Carlberg and Mannervik (1985). The reaction mixture (1 ml) contained 0.1 M HEPES pH 7.8, 1mM EDTA, 3 mM $MgCl_2$, 0.5 mM oxidized glutathione, 0.2 mM NADPH and 150 µl of the enzyme extract. The rate of NADPH oxidation was monitored by the decrease in absorbance at 340 nm for 2 min. Two blanks, one without the enzyme extract and the other without oxidized glutathione were used as controls.

1.3. Results

1.3.1. Plant biomass and symbiotic development

Shoot d. wt was higher in the mycorrhizal treatments (well-watered and drought stressed) than in the corresponding non-mycorrhizal controls (Table 1). Drought stress decreased shoot dry weight in all plants, but stressed mycorrhizal plants had 15% more shoot d. wt than stressed non-mycorrhizal treatment.

			Mycorrhizal infection		
Treatment	Shoot d. wt	Nodule f. wt	F	M	a
Br WW	2.40b	0.35c	0b	0b	0b
Br DS	2.00c	0.18d	0b	0b	0b
Br + Gm WW	2.90a	0.75a	95a	75a	81a
Br + Gm DS	2.30b	0.50b	94a	68a	88a

Table 1. Shoot dry weight (mg plant^{-1}), nodule fresh weight (g plant^{-1}) and mycorrhizal root infection (F%, M%, and a%) in non-mycorrhizal (Br) or mycorrhizal (Br+Gm) soybean plants grown under well-watered (WW) or drought stress (DS) conditions. Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple-range test ($n = 5$).

The nodule fresh weight was always higher in mycorrhizal than in non-mycorrhizal plants, regardless of the water treatment (Table 1).

No mycorrhizal colonization was observed in plants not provided with AM inoculum. The values of F, M and a were similar in both mycorrhizal treatments (unaffected by drought stress).

1.3.2. Nodule functioning and protein content

Nitrogenase activity was measured by the ARA test in total roots. ARA activity was higher in Gm+Br than in Br plants (Figure 1). This increase was considerably more evident under drought stress conditions (240% increase).

Nodules of mycorrhizal plants cultivated under well watered conditions had enhanced protein content as compared to the non-mycorrhizal plants (Figure 1). Drought decreased the protein content in nodules from the mycorrhizal plants, but it was similar to that of nodules from non-mycorrhizal plants.

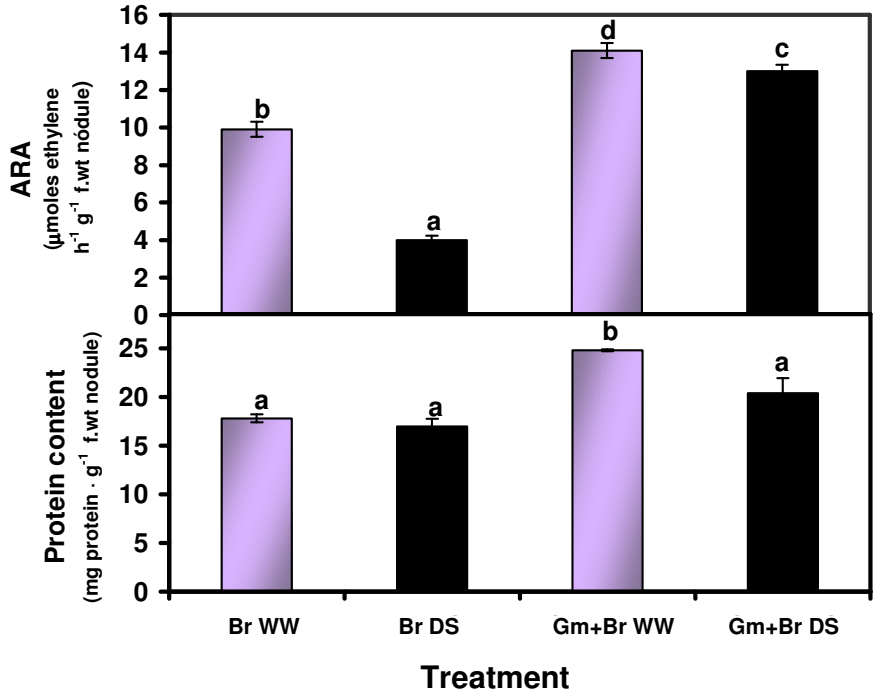


Figure 1. Acetylene reductase activity (ARA) (µmol ethylene h^{-1} g^{-1} f. wt nodule) and protein content (mg g f. wt^{-1} nodule) in nodules of soybean plants cultivated under well-watered (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm+Br, *Glomus mosseae* plus *B. japonicum*.

1.3.3. Hydrogen peroxide accumulation and oxidative damage to lipids

No significant differences among the different treatments were found in hydrogen peroxide concentration in nodules (Figure 2).

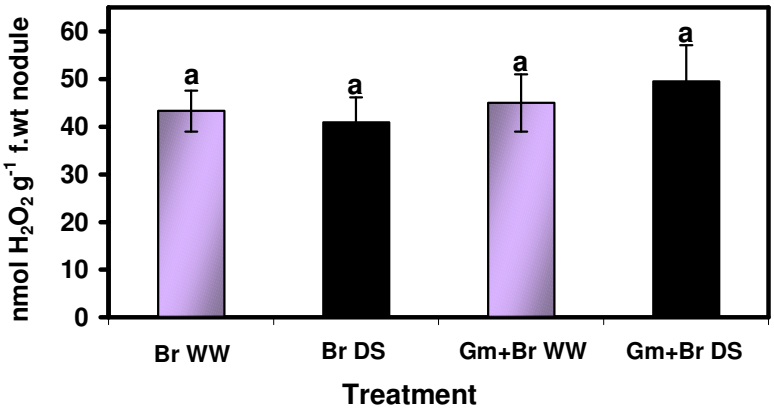


Figure 2. Hydrogen peroxide concentration (nmol H_2O_2 g^{-1} f. wt nodule) in nodules of soybean plants cultivated under well-watered (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm+Br, *Glomus mosseae* plus *B. japonicum*.

In roots, the oxidative damage to lipids increased as consequence of drought (Figure 3).

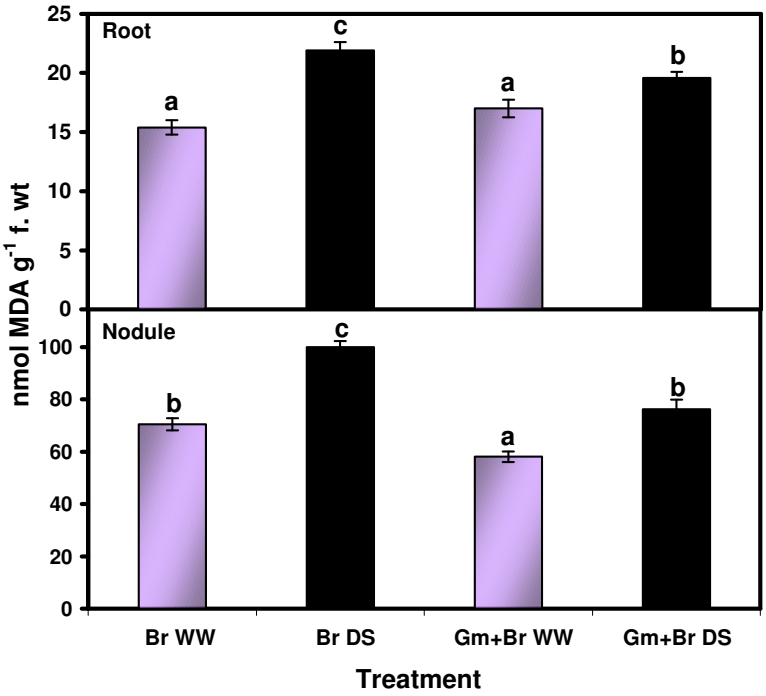


Figure 3. Oxidative damage to lipids (nmol MDA g^{-1} f. wt) in roots and nodules of soybean plants cultivated under well-watered (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm+Br, *Glomus mosseae* plus *B. japonicum*.

The increase was higher in non-mycorrhizal plants (by 42%) than in the mycorrhizal ones (by 15%). The oxidative damage to lipids was also higher in nodules from non-mycorrhizal plants than in those from mycorrhizal plants and that happened both under well-watered and under drought stress conditions. However, the differences in lipid peroxidation between nodules of mycorrhizal and non-mycorrhizal plants were higher under drought stress conditions (31% more lipid peroxidation in non-mycorrhizal plants) than under well-watered conditions (21% more lipid peroxidation in non-mycorrhizal plants).

1.3.4. Antioxidant activities

The specific SOD activity in roots was similar in all treatments. In contrast, in nodules, SOD activity was higher in non-mycorrhizal than in mycorrhizal plants (Figure 4A).

CAT activity in roots was 60% higher in mycorrhizal than in non-mycorrhizal plants under well-watered conditions (Figure 4B). Non-mycorrhizal plants subjected to drought showed the highest CAT activity in nodules.

APX in roots was enhanced by drought stress both in mycorrhizal and in non-mycorrhizal plants (Figure 4C). The highest APX activity was found in droughted non-mycorrhizal plants, which showed an increase of 80% relative to

the droughted mycorrhizal plants. In nodules, however, the highest APX activity was found in well-watered non-mycorrhizal plants. Drought decreased APX activity by 23% in such treatment. The lowest APX activity was found in nodules from well-watered mycorrhizal plants although drought increased the APX activity in AM plants to a level similar to that of droughted non-mycorrhizal plants.

The most significant result was obtained regarding GR activity (Figure 4D). In roots, mycorrhizal plants had higher GR activity than non-mycorrhizal ones (over 350% increase). Drought had no significant effect on GR activity of roots of either treatment. In nodules, again the mycorrhizal treatment had more GR activity than the non-mycorrhizal ones. The difference was more evident under drought stress conditions, where the increase in GR activity reached 534%.

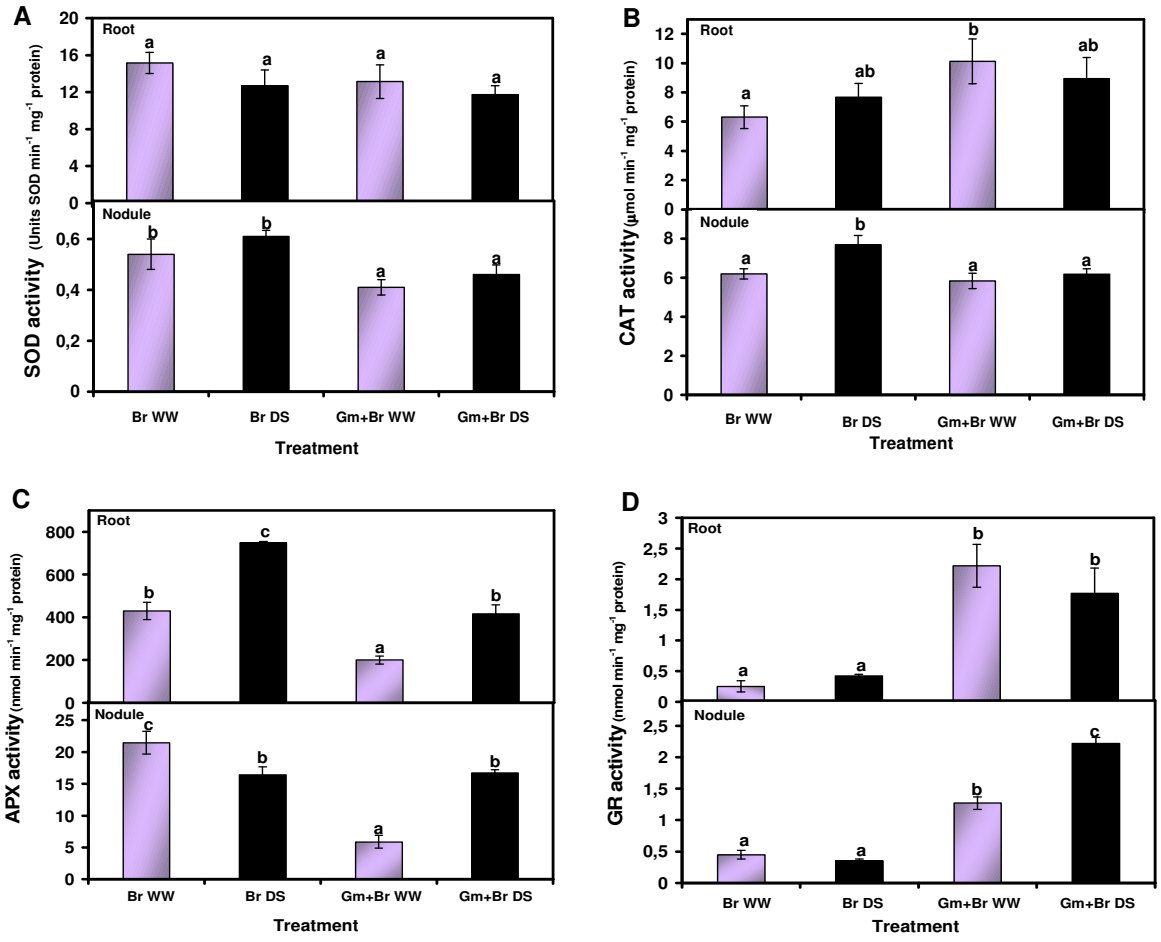


Figura 4. A. Superoxide dismutase (SOD) activity (units min^{-1} mg^{-1} protein); **B**. Catalase (CAT) activity (µmol min^{-1} mg^{-1} protein); **C**. Ascorbate peroxidase (APX) activity (nmol min^{-1} mg^{-1} protein);**D**. Gluthation reductase (GR) activity (nmol min^{-1} mg^{-1} protein). All of these determinations ware made in roots and nodules of soybean plants cultivated under well-water (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm+Br, *Glomus mosseae* plus *B. japonicum*.

1.3.5. Water consumption

The daily water consumption was measured with the Thetaprobe ML2x by determining the volumetric soil moisture in each pot. Since pots were daily watered to reach 17% of volumetric soil moisture (well-watered treatments) or 12% of volumetric soil moisture (drought stressed treatments), the reading of the ThetaProbe ML2x before watering was used to estimate water consumption per plant and per day. Under well-watered conditions, no differences in soil moisture were observed between mycorrhizal and non-mycorrhizal plants (Figure 8). In contrast, under drought stress conditions mycorrhizal plants depleted more the soil water content (average volumetric soil moisture of 7.55%) than non-mycorrhizal plants (average volumetric soil moisture of 8.15%). Such difference in volumetric soil moisture is equivalent to 3 ml pot^{-1} day^{-1} of extra water consumption in mycorrhizal plants.

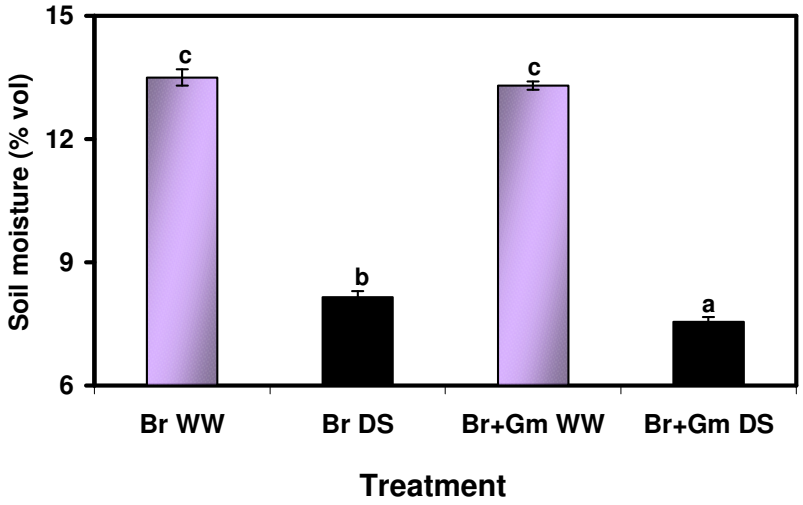


Figure 5. Volumetric soil moisture (% vol) before watering in pots containing soybean plants cultivated under well-watered (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm+Br, *Glomus mosseae* plus *B. japonicum*.

1.4. Discussion

Many of the degenerative reactions associated with several biotic, abiotic and xenobiotic stresses are mediated by ROS. The term ROS is generic, embracing not only free radicals such as superoxide and hydroxyl radicals, but also H_2O_2 and singlet oxygen. While it is generally assumed that the hydroxyl radical and singlet oxygen are so reactive that their production must be minimized (Jakob and Heber, 1996), $O_2^{\cdot -}$ and H_2O_2 are synthesized at very high rates even under optimal conditions (Noctor and Foyer, 1998). The chief toxicity of $O_2^{\cdot -}$ and H_2O_2 is thought to reside in their ability to initiate cascade reactions that result in the production of the hydroxyl radicals. These radicals (and their derivatives) are among the most reactive species known to chemistry, capable of reacting indiscriminately to cause oxidative damage to biomolecules such as lipid

peroxidation, denaturation of proteins and mutation of DNA (Halliwell and Gutteridge, 1989; Bowler *et al.*, 1992).

We concluded in our previous study that alleviation of oxidative damage to biomolecules in nodules might be involved in the protective effect of AM symbiosis against nodule senescence (Ruiz-Lozano *et al.,* 2001). In this study we have evidenced again a significantly lower oxidative damage to lipids in roots and nodules of droughted mycorrhizal plants than in non-mycorrhizal plants. Hence, in this investigation we analyzed in root and nodule tissues the activities of four enzymes well documented as involved in ROS scavenging.

The efficient destruction of $O_2^{\cdot-}$ and H_2O_2 requires the action of several antioxidant enzymes acting in synchrony. Superoxide is rapidly converted to H_2O_2 by the action of SOD (Bowler *et al.,* 1992). However, dismutation of $O_2^{\cdot-}$ simply serves to convert one destructive ROS to another. Since H_2O_2 is a strong oxidant that rapidly oxidizes thiol groups, it cannot be allowed to accumulate to excess (Noctor and Foyer 1998). CATs convert H_2O_2 to water and molecular oxygen in peroxisomes. These enzymes have extremely high maximum catalytic rates but low substrate affinities (Willekens *et al.,* 1995). Furthermore, the absence of CAT in the chloroplast precludes a role in protection of the thiol-regulated enzymes of the Calvin cycle. An alternative mode of H_2O_2 destruction is via peroxidases, which are found throughout the cell and which have a much higher affinity for H_2O_2 than CAT (Jiménez *et al.,* 1997). Plants contain high activities for the enzymes of the ascorbate-glutathione cycle in which H_2O_2 is scavenged. In the first step of this pathway, APX, which is the most important peroxidase in H_2O_2 detoxification (Noctor and Foyer, 1998), catalyzes the reduction of H_2O_2 to water by ascorbate, and the resulting monodehydroascorbate and dehydroascorbate are reduced back to ascorbate by monodehydroascorbate reductase (MR) and by dehydroascorbate reductase (DR) plus GR, respectively (Iturbe-Ormaetxe *et al.,* 2001).

Curiously, the hydrogen peroxide concentration measured in nodules in this study was similar in all treatments. However, it should be considered that H_2O_2 is involved in virtually all major areas of aerobic biochemistry (e.g. respiratory and photosynthetic electron transport; oxidation of glycolate, xanthine and glucose) and is produced in copious quantities by several enzyme systems (Noctor and Foyer, 1998). Moreover, in some circumstances, the destructive power and signalling potential of ROS such as H_2O_2 are utilized as an effective means of defense (Levine *et al.,* 1994; Foyer *et al.,* 1997).

The results obtained in relation to four antioxidant activities showed that only the GR activity was higher in mycorrhizal roots than in the non-mycorrhizal ones. The other antioxidant activities were similar (SOD and CAT) or lower (APX) in the droughted mycorrhizal roots than in the corresponding non-mycorrhizal ones. Similarly, in nodules, the SOD, CAT and APX activities were lower in the droughted mycorrhizal plants than in the non-mycorrhizal plants while, again, the GR activity was higher in nodules from mycorrhizal plants. In previous works where several enzymes have been studied under the same stress condition differential responses have frequently been observed (Walker and McKersie, 1993; Willekens *et al.,* 1994; Conklin and Last, 1995). Moreover, determinations of total activities in crude

homogenates may not adequately reflect the importance of compartment-specific changes (Noctor and Foyer, 1998) since it is not considering the activities at the sites where antioxidants are synthesized and required, i.e. the various nodule and root cell organelles (Mittova *et al.,* 2000).

In all living organisms, glutathione (GSH) is the major low molecular weight thiol-containing compound. It is present in millimolar concentrations in different tissues, where it is a general reductant (Foyer *et al.,* 1991). In addition, it has several important functions, including the removal of toxic oxygen derivatives in the ascorbate-glutathione cycle, the induction of enzymes, and it participates in sulphur metabolisms and gene expression (Foyer *et al.,* 1995). Some legumes contain homogluthatione (hGSH) instead of or in addition to GSH. It is not known why hGSH is restricted to legumes and what specific role, if any, this thiol may play in nodule functioning. Matamoros *et al.* (1999) measured the thiol composition in 8 legumes of agronomic interest, including soybean. This study showed that hGSH predominated in soybean, bean and mungbean plants. In any case, both thiol compounds (GSH and hGSH) were present in nodules of these plants. The same study also showed that, under induced premature nodule senescence, the concentration of GSH in nodules of bean became higher than that of hGSH, while no measurement was done in soybean.

Although it has been suggested that enhanced GSH content is not in itself sufficient to improve resistance to high rates of ROS production (Noctor and Foyer, 1998), the increase of GR activity (the enzyme which regenerates the oxidized GSH into its reduced form) observed in mycorrhizal plants could be of importance regarding alleviation of damage caused by drought stress. In a recent study Kranner (2002) correlated the amount of reduced GSH with different degrees of desiccation tolerance in lichens. GR has been postulated to play an important role in plant protection against various forms of stress (Aono *et al.,* 1995). Enhanced GR activity has been associated with increases in ascorbate contents (Foyer *et al.,* 1995), better protection of ascorbate and glutathione pools against paraquat stress (Foyer *et al.,* 1991; 1995), decreased sensitivity to photoinhibition (Foyer *et al.,* 1995), and mitigated foliar damage during exposure to paraquat (Aono *et al.,* 1993). Moreover, the decrease in GR activity has been correlated with enhanced paraquat sensitivity in tobacco plants (Aono *et al.,* 1995).

In conclusion, the results obtained in this study suggest that the consistently higher GR activity in roots and nodules of mycorrhizal plants might have generated reduced antioxidants (GSH), which helped to decrease the oxidative damage to biomolecules that is involved in premature nodule senescence. In fact, the ASC-GSH cycle, of which the GR together with APX are important components, is one of the main antioxidant defenses in plants (Becana *et al.,* 2000) and is also present in the cytosol of nodule cells (Dalton, 1995). It has been proposed that the impairment of the ASC-GSH cycle in the nodules may be a common feature in the process of nodule senescence (Escuredo *et al.,* 1996). This idea agrees with recent findings by Burritt et al. (2002) who have postulated that the ability of seaweeds plants to prevent or reduce the production of ROS was due to increased activity of the enzymes required to regenerate ascorbate and

glutathione, as is the case of GR. However, we would also expect an increased APX activity in nodules of droughted mycorrhizal plants, while it was similar to that found in nodules of droughted non-mycorrhizal plants. Hence, it seems unlikely that only the increase of GR activity is responsible of the important protective effect observed (Ruiz-Lozano *et al.,* 2001). We propose that other mechanisms might have contributed to the protection of soybean plants against drought-induced nodule senescence. One possibility is that AM symbiosis leads to a lower drought-induced oxidative stress in mycorrhizal plants due to primary drought-avoidance mechanisms such as the higher water retention properties of a mycorrhizal soil as compared to the soil of non-mycorrhizal plants (Augé *et al.,* 2001a, b), as well as to the ability of AM hyphae to take up water from sources inaccessible to the non-mycorrhizal roots and transfer to the host plant (Hardie, 1985; Ruiz-Lozano and Azcón, 1995). Data on daily plant water consumption showed a higher ability of mycorrhizal plants to deplete soil water when water availability was limited and support the hypothesis that a primary drought-avoidance mechanisms, namely direct water uptake by hyphae, is also involved in the protection of mycorrhizal soybean plants against premature drought-induced nodule senescence.

1.5. Acknowledgements

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INFLUENCIA DE LA SIMBIOSIS MICORRÍCICO ARBUSCULAR (MA) SOBRE EL POTENCIAL HÍDRICO. ACUMULACIÓN DE SOLUTOS Y ESTRÉS OXIDATIVO EN PLANTAS DE SOJA SOMETIDAS A DÉFICIT HÍDRICO

Resumen

Este estudio ha investigado varios aspectos relacionados con la tolerancia a la sequía en plantas de soja micorrizadas. La investigación ha incluido tanto parte aérea como raíz con el fin de poder revelar el tejido diana donde se manifiestan los efectos de la MA frente al estrés hídrico. Las plantas de soja micorrizadas y no micorrizadas fueron cultivadas tanto en condiciones óptimas como en condiciones de déficit hídrico, y se determinaron parámetros como el estatus hídrico de la hoja, acumulación de solutos y daño oxidativo a lípidos. Los resultados mostraron que las plantas MA se vieron protegidas frente a la sequía, como lo demuestra su mayor producción de biomasa de parte aérea. El potencial hídrico de la hoja fue también mayor en plantas MA estresadas (-1.9 MPa) que en las plantas no micorrizadas (-2.5 MPa). Las raíces MA acumularon más prolina que las raíces no MA, mientras que se observó el efecto contrario en parte aérea. La peroxidación lipídica fue un 55% menor en parte aérea de plantas micorrizadas sometidas a estrés hídrico que en las plantas no micorrizadas sometidas a estrés. Dado que no se encontró correlación directa entre el menor daño oxidativo a lípidos en plantas MA y la actividad de los enzimas antioxidantes, parece que la simbiosis MA primero mejoró el ajuste osmótico en raíces, lo que podría haber contribuido a mantener un gradiente de potencial hídrico favorable a la entrada de agua desde el suelo a la raíz. Ello permitirá un mayor potencial hídrico en las hojas de plantas MA durante la sequía y las protegería frente al estrés oxidativo generado por el déficit hídrico. Estos efectos resultarían en un incremento de la tolerancia de las plantas MA frente a la sequía.

Palabras clave: Simbiosis micorrícico arbuscular, sequía, ajuste osmótico, daño oxidativo.

ARBUSCULAR MYCORRHIZAL INFLUENCE ON LEAF WATER POTENTIAL, SOLUTE ACCUMULATION, AND OXIDATIVE STRESS IN SOYBEAN PLANTS SUBJECTED TO DROUGHT STRESS

Abstract

This study investigated several aspects related to drought tolerance in arbuscular mycorrhizal (AM) soybean plants. The investigation included both shoot and root tissues in order to reveal the preferred target tissue for AM effects against drought stress. Non-AM and AM soybean plants were grown under well-watered or drought-stressed conditions, and leaf water status, solute accumulation, oxidative damage to lipids and other parameters were determined. Results showed that AM plants were protected against drought, as evidenced by their significantly higher shoot biomass production. The leaf water potential was also higher in stressed AM plants (-1.9 MPa) than in non-AM plants (-2.5 MPa). The AM roots had accumulated more proline than non-AM roots, while the opposite was observed in shoots. Lipid peroxides were 55% lower in shoots of droughted AM plants than in droughted non-AM plants. Since there was no correlation between the lower oxidative damage to lipids in AM plants and the activity of antioxidant enzymes, it seems that first the AM symbiosis enhanced osmotic adjustment in roots, that could contribute to maintain a water potential gradient favorable to the water entrance from soil into the roots. This enabled higher leaf water potential in AM plants during drought and kept the plants protected against oxidative stress, and these cumulative effects increased the plant tolerance to drought.

Key words: *arbuscular mycorrhizal symbiosis, drought, osmotic adjustment, oxidative damage.*

2.1. Introduction

Drought stress is considered one of the most important abiotic factors limiting plant growth and yield in many areas (Kramer and Boyer, 1997) and arbuscular mycorrhizal (AM) symbiosis can protect host plants against its detrimental effects (for reviews see Augé, 2001, Ruiz-Lozano, 2003). Although mycorrhizal effects on plant water relations are not as dramatic and consistent as those on P acquisition and host growth, it is accepted that modest changes, if sustained, can have meaningful effects on plant fitness (Augé, 2001). Several studies on the 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 (Ruiz-Lozano, 2003). 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 (Augé *et al.*, 2001a). 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 enhanced osmotic adjustment and leaf hydration or reduced oxidative damage caused by the reactive oxygen species (ROS) generated during drought (Ruiz-Lozano, 2003). In fact, it has been shown that mycorrhizal colonization and drought interact in modifying free amino acid and sugar pools in roots (Augé *et al.*, 1992). A greater osmotic adjustment has been also reported in leaves of mycorrhizal basil plants than in nonmycorrhizal ones during a lethal drought period (Kubikova *et al.*, 2001). In the same way, AM plants had postponed declines in leaf water potential (Ψ) during drought stress (Davies *et al.*, 1992; Subramanian *et al.*, 1997; El-Tohamy *et al.*, 1999) and leaf Ψ returned to non-AM level more quickly in AM than non-AM maize plants after the relief of drought (Subramanian *et al.*, 1997). In contrast, leaf Ψ was similar in AM and non-AM plants when water was not limiting (Ebel *et al.*, 1996; Bryla and Duniway, 1997). Finally, mycorrhizal lettuce plants showed increased superoxide dismutase (SOD) activity under drought stress and this correlated to plant protection against drought (Ruiz-Lozano *et al.*, 1996; 2001a). In the same way, AM soybean plants subjected to drought had lower oxidative damage to lipids and proteins in nodules than non-AM plants, and this was linked with protection against nodule senescence (Ruiz-Lozano *et al.*, 2001b; Porcel *et al.*, 2003).

While several studies have focussed on the physiological, biochemical and molecular responses of shoot plant tissues to drought, few studies have considered the root systems at the same time, although this organ constitutes the primary site of drought perception and plays an important role in drought stress resistance and recovery. Root size and architecture are important factors for determining yield performance, particularly under conditions of limited water availability (Price *et al.*, 2000). Roots are also actively involved in the perception and transduction of the drought stress signal and the information seems to be transferred to the whole plant via growth regulators (ABA or ethylene) that induce stomatal closure (Dubos and Plomion, 2003).

This study extends to the root system previous investigations on soybean shoot and nodule tissues in order to elucidate the preferred target tissue for AM effects against drought stress. For that, the effects of AM symbiosis on solute accumulation and on the oxidative damage to lipids and antioxidant activities in root and shoots of soybean plants were evaluated.

2.2. Materials and Methods

2.2.1. Experimental design and statistical analysis

The experiment consisted of a randomized complete block design with two inoculation treatments: (1) control nonmycorrhizal plants (2) plants inoculated with the mycorrhizal fungus *Glomus intraradices* Schenck and Smith. Ten replicates of each treatment were done totalling 20 pots (one plant per pot) so

that half of them were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 10 days before harvest.

Data were subjected to analysis of variance (ANOVA) with mycorrhizal treatment, water supply and mycorrhizal 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.

2.2.2. 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 days). The soil had a pH of 8.1 (water, 10 g soil in 25 ml water); 1.81% organic matter, available 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_2O_2 solution for 8 min, then washed several times with sterile water to remove any trace of chemical that could interfere in 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 the sterilized soil/sand mixture. A suspension (1 ml $seed^{-1}$) of the diazotrophic bacterium *Bradyrhizobium japonicum*, strain USDA 110 (10^9 cell ml^{-1}), was sprinkled over all seedlings at the time of planting.

Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted of soil, spores, mycelia and colonized root fragments. The AM species was *Glomus intraradices* Schenck and Smith, isolate EEZ 6, BEG 121. Ten grams of inoculum were added to the appropriate pots at sowing time just below soybean seedlings.

2.2.3. 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 using a pressure plate apparatus and applying a pressure of one third atmosphere for 48h, and then determining the volumetric soil moisture) during the first 5 weeks of plant growth. At this time half of the plants were allowed to dry until soil water content reached 70% field capacity (three days needed), which corresponded to 10% volumetric soil moisture (also determined experimentally in a previous assay). Plants were maintained under such conditions for additional 10 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 24h-period comprised between each rewatering the soil water content was progressively decreasing until a minimum value of 55% of field capacity.

Each week throughout the experiment, plants received 10 ml of Hewitt´s nutrient solution lacking N and P (Hewitt, 1952). Three weeks after planting, non-AM plants received nutrient solution amended with 0.35 mM K_2HPO_4 (Goicoechea *et al.*, 1997). That P concentration was chosen to obtain well-watered plants of similar size and P content in both plant treatments.

2.2.4. Parameters measured

2.2.4.1. Biomass production

At harvest (48 days after planting), the shoot and root system were separated and the shoot dry weight (DW) measured after drying in a forced hot-air oven at 70 ºC for two days.

2.2.4.2. Symbiotic development

The percentage of mycorrhizal root infection was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (v/v), according to Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980).

2.2.4.3. Leaf water potential

The mid-day leaf water potential (Ψ) was determined with a C-52 thermocouple psychrometer chamber and a HR-33T dew point microvoltmeter (Wescor Inc, Logan, UT, USA). Leaf discs corresponding to the third youngest leave were cut, placed inside the psychrometer chamber and allowed to reach temperature and water vapour equilibrium for 30 min before measurements were made by the dew point method.

2.2.4.4. Proline and total soluble sugars

Free proline and total soluble sugars were extracted from 1 g of fresh root and leaves (Bligh and Dyer, 1959). The methanolic phase was used for quantification of both substances. Proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates *et al.* (1973). Soluble sugars were analyzed by 0.1 ml of the alcoholic extract reacting with 3 ml freshly prepared anthrone (200 mg anthrone + 100 ml 72% (w:w) H_2SO_4) and placed in a boiling water bath for 10 min according to Irigoyen *et al.* (1992). After cooling, the absorbance at 620 nm was determined in a Shimadzu UV-1603

spectrophotometer. The calibration curve was made using glucose in the range of 20 to 400 μg ml^{-1}.

2.2.4.5. Hydrogen peroxide concentration and oxidative damage to lipids

For the determination of hydrogen peroxide concentration aliquots (0.5 g) of roots and leaves were homogeneized with an ice-cold potter in HCl 25 mM and filtered through four layers of nylon cloth. The supernatants were adjusted to pH 7.0 for subsequent H_2O_2 quantification, which was performed by the 4-aminoantipyrine method (Frew *et al.*, 1983).

Lipid peroxides were extracted by grinding 0.5 g of roots or leaves with and ice-cold mortar and 6 ml of 100 mM potassium phosphate buffer (pH 7). Homogenates were filtered through one Miracloth layer and centrifuged at 15,000 g for 20 min. The chromogen was formed by mixing 200 µl of supernatants with 1 ml of a reaction mixture containing 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) 2-thiobarbituric acid (TBA), 0.1% (w/v) butyl hydroxytoluene, 0.25 N HCl and by incubating the mixture at 100 °C for 30 min (Minotti and Aust, 1987). After cooling at room temperature, tubes were centrifuged at 800 g for 5 min and the supernatant was used for spectrophotometric reading at 535 nm. Lipid peroxidation was estimated as the content of 2-thiobarbituric acid-reactive substances (TBARS) and expressed as equivalents of malondialdehyde (MDA) according to Halliwell and Gutteridge (1989). The calibration curve was made using MDA in the range of 0.1-10 nmol. A blank for all samples was prepared by replacing the sample with extraction medium, and controls for each sample were prepared by replacing TBA with 0.25 N HCl. In all cases, 0.1% (w/v) butyl hydroxytoluene was included in the reaction mixtures to prevent artifactual formation of 2-thiobarbituric acid-reactive substances (TBARS) during the acid-heating step of the assay.

2.2.4.6. Preparation of extracts from roots and shoots

Enzymes were extracted at 0-4 °C from 1 g (fresh weight (FW)) of root or shoot tissues using a mortar and pestle with 50 mg polyvinylpolypyrrolidone (PVPP) and 10 ml of the following optimized medium: 50 mM K-phosphate buffer pH 7.8 containing 0.1 mM EDTA for superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Gogorcena *et al.*, 1995). The same medium supplied with 10 mM β-mercaptoethanol was used for glutathione reductase (GR) (Moran *et al.*, 1994). Extracts were filtered through four layers of nylon cloth and centrifuged at 20,000 g, 20 min, 0-4 °C. The supernatants were kept at –70 °C for subsequent enzymatic assays.

Soluble protein was determined by the dye binding microassay (Bio-Rad, Madrid, Spain) using BSA as the standard (Bradford, 1976).

2.2.4.7. Enzyme assays

Total SOD activity (EC 1.15.1.1) was measured according to Beyer and Fridovich (1987) based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25 °C. CAT activity (EC 1.16.1.6) was measured by the disappearance of H_2O_2 (Aebi, 1984). The reaction mixture (3ml) contained 10.6 mM H_2O_2. The reaction was initiated by adding 25 µl of the extract and monitoring the change in absorbance at 240 nm and 25 °C for 3 min. APX activity (EC 1.11.1.11) was measured in a 1 ml reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM hydrogen peroxide and 0.5 mM ascorbate. Adding the H_2O_2 started the reaction and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate of ascorbate (Amako *et al.*, 1994). Finally, GR activity (EC 1.6.4.2) was determined by the procedure of Carlberg and Mannervik (1985). The reaction mixture (1 ml) contained 0.1 M HEPES pH 7.8, 1mM EDTA, 3 mM $MgCl_2$, 0.5 mM oxidized glutathione, 0.2 mM NADPH and 150 µl of the enzyme extract. The rate of NADPH oxidation was monitored by the decrease in absorbance at 340 nm for 2 min. Two blanks, one without the enzyme extract and the other without oxidized glutathione were used as controls.

2.3. Results

2.3.1. Plant biomass and mycorrhizal colonization

Under well-watered conditions, shoot DWs of AM and non-AM soybean plants were similar (Table 1).

Treatment	Shoot DW[a] (g plant^{-1})	Root DW[a] (g plant^{-1})	Leaf Ψ[a] (MPa)
AM plants			
Well-watered	1.20a	0.95a	-1.5c
Drought-stressed	0.70b	0.57b	-1.9b
Non-AM plants			
Well-watered	1.27a	1.11a	-1.6c
Drought-stressed	0.55c	0.50b	-2.5a
Significance of sources of variation			
Mycorrhizae (M)	**	ns	*
Water regime (W)	***	**	***
M x W	*	ns	*

Table 1. Shoot and root dry weight (g^{-1} plant) and mid-day leaf water potential (Ψ, MPa) in AM or non-AM soybean plants grown under well-watered or drought-stressed conditions.

[a] Means followed by the same letter are not significantly different (P < 0.05) as determined by Duncan's multiple-range test (n = 5). Significance of the sources of variation is also displayed. * P≤ 0.05; ** P≤ 0.01; * **P≤ 0.001; ns, not significant.

Drought stress decreased plant growth in both treatments (a decrease of 57% in non-AM plants and 42% in AM plants). In any case, drought-stressed AM plants showed enhanced shoot DW (27%) as compared to non-AM plants. Root DW was similar in AM and non-AM soybean plants at whatever water regime.

No mycorrhizal colonization was observed in plants not provided with AM inoculum. Mycorrhizal plants showed about 50% of mycorrhizal root length both under well-watered and drought-stressed conditions.

2.3.2. Leaf water potential (Ψ)

The leaf Ψ determined at the end of the drought period was similar in AM and non-AM plants cultivated under well-watered conditions (Table 1). Drought stress decreased Ψ, but the decrease was larger in non-AM plants (–2.5 MPa) than in AM plants (–1.9 MPa). The time-course of leaf Ψ during the entire drought period showed a similar pattern for AM and non-AM plants, both under well-watered and under drought stress conditions, with droughted non-AM plants always exhibiting lower leaf Ψ than the corresponding AM plants (Figure 1).

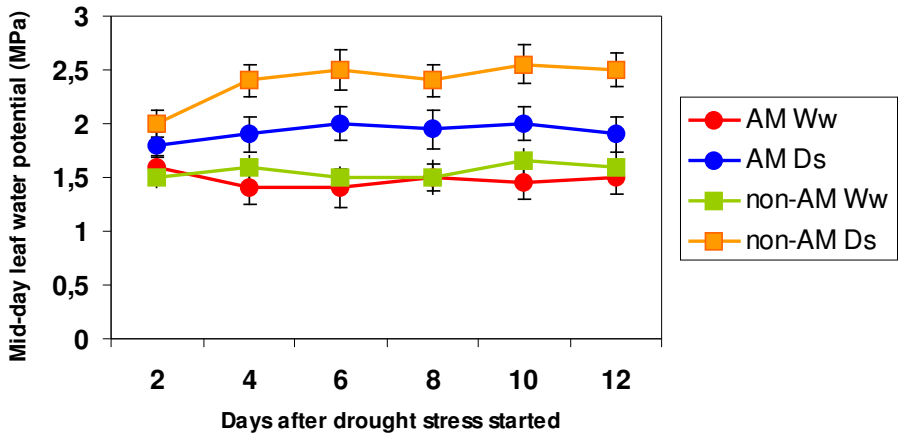

Figure 1. Time-course of midday leaf water potential (MPa) in AM or non-AM soybean plants cultivated under well-watered (Ww) or under drought stressed (Ds) conditions. The measurements were taken during the period that half of plants were subjected to drought stress.

2.3.3. Proline and total soluble sugar accumulation

Accumulation of proline increased considerably in roots as consequence of drought stress and AM plants accumulated 14% more proline in roots than non-AM plants (Figure 2; Table 2). In shoots drought stress also induced the accumulation of proline. However, in such plant tissue, AM plants accumulated 39% less proline than non-AM plants.

	Proline		SS		ODL	
	Root	Shoot	Root	Shoot	Root	Shoot
Mycorrhizae (M)	*a	*	*	*	**	*
Water regime (W)	***	***	**	*	*	*
M x W	*	*	ns	ns	ns	ns

Table 2. Significance of the sources of variation for proline and soluble sugar (SS) contents, and for oxidative damage to lipids (ODL).

* P≤ 0.05; ** P≤ 0.01; * **P≤ 0.001; ns, not significant.

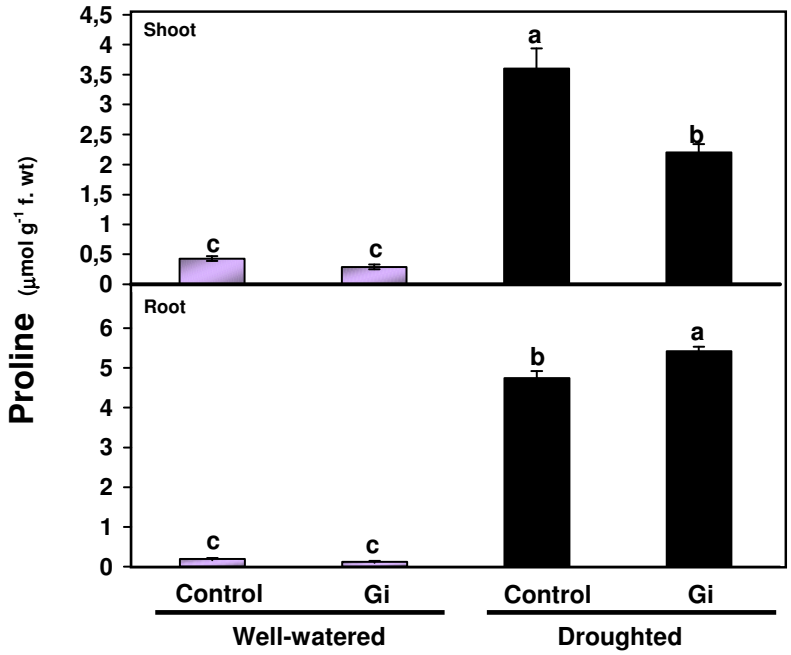

Figure 2. Proline content (µmol g^{-1} FW) in shoots and roots of AM or non-AM soybean plants cultivated under well-watered or under drought stress conditions

Under well-watered conditions, total soluble sugars in roots were higher in AM plants than in non-AM plants (Figure 3; Table 2).

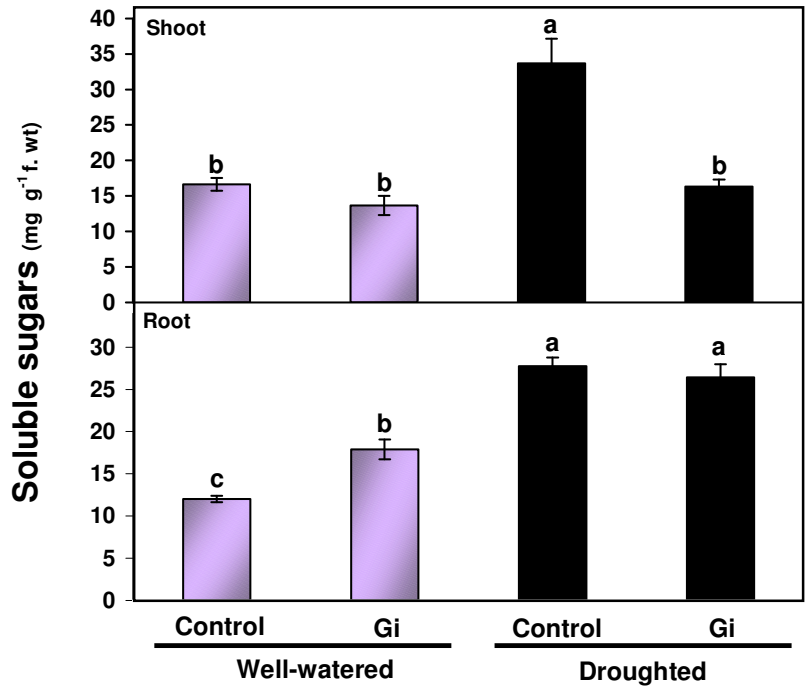

Figure 3. Soluble sugars content (mg g^{-1} FW) in shoots and roots of AM or non-AM soybean plants cultivated under well-watered or under drought stress conditions.

Drought stress increased sugar accumulation, in both treatments, but no significant differences between treatments were found under such conditions according to ANOVA. In shoots, total soluble sugar was similar in both treatments under well-watered conditions. Drought increased the sugar content in non-AM plants by (116%), while AM plants showed similar sugar content than under well-watered conditions.

2.3.4. Hydrogen peroxide accumulation and oxidative damage to lipids

No significant differences among the different treatments were found in hydrogen peroxide concentration in roots or in shoots (data not shown). Roots of all treatments accumulated about 150 nmol H_2O_2 g^{-1} FW, while shoots accumulated about 10 times more.

In roots, the oxidative damage to lipids increased as consequence of drought only in non-AM plants (Figure 4; Table 2).

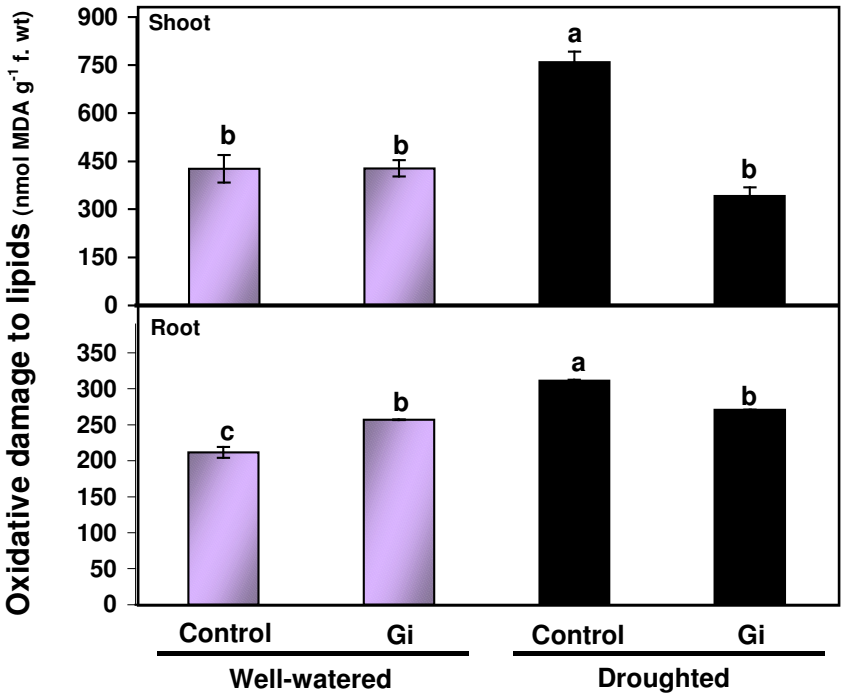

Figure 4. Oxidative damage to lipids (nmol MDA g^{-1} FW) in shoots and roots of AM or non-AM soybean plants cultivated under well-watered or under drought-stressed conditions.

AM plants showed similar level of lipid peroxidation under both water conditions. However, under drought conditions, roots of AM plants exhibited 13% less lipid peroxides than roots of non-AM plants. In shoots, the different behaviour of AM and non-AM plants was more evident. Drought enhanced lipid peroxidation in non-AM plants by 78%, while lipid peroxidation in shoots of AM plants remained unaffected. In any case, under drought conditions, shoots of AM plants had 55% less lipid peroxides than shoots of non-AM plants.

2.3.5. Antioxidant activities

In roots, SOD activity was similar in the different treatments, except for drought-stressed AM roots, which had significantly lower SOD (Table 3). In shoots, AM plants had lower SOD activity than non-AM plants when cultivated under well-watered conditions and higher activity when cultivated under drought stress conditions.

CAT activity showed an opposite behaviour in roots and in shoots (Table 3). In roots, CAT activity only increased in AM plants as consequence of drought. In shoots the CAT activity of AM plants was higher than in non-AM plants under well-watered conditions, but under drought stress conditions the CAT activity of AM plants decreased, reaching a similar value than in non-AM plants.

APX resulted always higher in non-AM plants than in AM plants (Table 3). Drought stress increased the APX activity in shoots of both AM and non-AM plants in comparison to well-watered conditions. Mycorrhizal roots had significantly lower APX regardless of well-watered or drought-stressed conditions.

GR activity was notably enhanced by drought stress in roots of non-AM plants and decreased in roots of AM plants (Table 3). Drought-stressed AM and non-AM shoots had similar GR activities while under well-watered conditions, the GR activity increased by 40-fold in non-AM compared with AM plants.

Treatments	SOD[a]		CAT[a]		APX[a]		GR[a]	
	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
AM plants								
Well-watered	10.2a	1.6c	3.3b	2.4a	0.5c	6.9c	1.6b	0.2c
Drought-stressed	7.0b	2.4b	5.3a	1.2c	0.6c	18.1b	0.6c	0.9b
Non AM plants								
Well-watered	8.7a	2.9a	4.7ab	1.8b	1.6a	13.5b	1.5bc	4.0a
Drought-stressed	9.0a	1.5c	6.0a	1.4bc	1.2b	24.7a	3.3a	1.4b
Significance of sources of variation								
Mycorrhizae (M)	*	**	ns	*	**	**	*	*
Water regime (W)	*	***	*	*	*	**	*	*
M x W	ns	*	ns	ns	ns	*	ns	ns

Table 3. SOD, CAT, APX and GR activities in roots and shoots of AM or non-AM soybean plants grown under well-watered or drought-stressed conditions.

[a]Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple-range test ($n = 5$). Significance of the sources of variation is also displayed. * $P \leq 0.05$; ** $P \leq 0.01$; * **$P \leq 0.001$; ns, not significant.

2.4. Discussion

Water deficit has profound effects on crop production. Even plants with an optimum water supply experience transient water shortage periods, where water absorption cannot compensate for water loss by transpiration (Kramer and Boyer, 1997). Arbuscular mycorrhizal symbiosis has been shown to increase plant tolerance to water deficit, although the exact mechanisms involved are still a matter of debate (Augé, 2001; Ruiz-Lozano, 2003).

This study investigated physiological and biochemical aspects related to water relations and drought tolerance in AM and non-AM plants subjected to drought stress. AM plants showed higher tolerance to the drought stress imposed (only for 10 d) than non-AM plants, as shown by their enhanced shoot biomass production (27%), higher (less negative) leaf Ψ under such conditions or lower lipid peroxidation.

Drought stressed plants have been shown to accumulate organic osmolytes such as sugars and amino acids (proline) that are known to contribute to the host plant tolerance under water-deficit conditions (Schellembaum *et al.*, 1998; Trotel-Aziz *et al.*, 2000). The enhanced sugar content in AM roots under well-watered conditions may be due to the sink effect of the mycorrhizal fungus demanding

sugars from shoots tissues. Under drought the sugar content in roots was similar in both treatments, suggesting that osmotic adjustment occurred. In contrast, in shoots the sugar content of droughted AM plants was considerably lower than in non-AM plants. Schellembaum *et al.* (1998) suggested that the AM fungus can be a strong competitor for root-allocated carbon under conditions limiting photosynthesis. These authors proposed that the lower hexose accumulation in leaves of mycorrhizal plants in drought could be due to a lower availability of photosynthates for storage in these tissues. However, another explanation is also possible, that AM shoots were less strained by drought than non-AM ones. The lower accumulation of compatible solutes may indicate that the plants more successfully avoided drought stress (Augé, 2001). In fact, proline, the other osmoregulator measured in this study, also accumulated less in shoots of AM plants than in non-AM plants. The higher leaf Ψ in stressed AM plants (-1.9 MPa) than in non-AM plants (-2.5 MPa) also support this last hypothesis (Subramanian *et al.*, 1995). In contrast, in roots proline accumulated more in AM plants than in non-AM ones. The accumulation of proline and total soluble sugar in roots could have provided the root with an osmotic mechanism to maintain a favourable Ψ gradient for water entrance into the roots (Irigoyen *et al.*, 1992) leading, therefore, to a lower stress injury in the plant.

In addition to acting as an osmoprotectant, proline also serves as a sink for energy to regulate redox potentials, as a hydroxyl radical scavenger, as a solute that protects macromolecules against denaturation, and as a means of reducing acidity in the cell (Smirnoff, 1993; Kishor *et al.*, 1995). This can be important for AM plants since drought also induces an oxidative stress, which, it has been pointed out, is responsible for many of the degenerative reactions caused by drought. The oxidation of membrane lipids is a reliable indication of uncontrolled free radical production and hence of oxidative stress (Noctor and Foyer, 1998). Accordingly, the amount of lipid peroxides was quantified in roots and shoots. In roots, the lipid peroxidation in AM plants subjected to drought was 13% lower than in droughted non-AM plants. In shoots, lipid peroxidation was 55% lower in droughted AM plants than in droughted non-AM plants. Curiously, the hydrogen peroxide concentration measured in this study was similar in all treatments. However, it should be remembered that H_2O_2 is involved in virtually all major areas of aerobic biochemistry (e.g. respiratory and photosynthetic electron transport; oxidation of glycolate, xanthine and glucose) and is produced in copious quantities by several enzyme systems, even under optimal conditions (Noctor and Foyer, 1998). Moreover, in some circumstances, the destructive power and signalling potential of ROSs such as H_2O_2 are utilized as an effective means of defence (Levine *et al.*, 1994; Foyer *et al.*, 1997).

The activities of four antioxidant enzymes were measured for correlation with the oxidative damage to lipids. Results showed that there was no relationship between the antioxidant activities and the decrease in lipid peroxidation in roots and shoots of droughted AM plants. In addition, only shoot SOD and shoot APX activities showed a significant interaction between mycorrhization and water regime, while no significant interaction was observed for the other activities. In

general, the results obtained for the four antioxidant activities agree with previous results obtained in roots of soybean plants inoculated with *G. mosseae* (Porcel *et al.*, 2003). The only exception was found in relation to the GR activity that, in the present study involving *G. intraradices*, was lower in roots of droughted AM plants than in the corresponding non-AM plants, while in the previous study, involving *G. mosseae*, the GR activity increased in AM plants (Porcel *et al.*, 2003). However, it must be borne in mind that the AM fungi utilized in both studies were different and that dissimilar behaviour of AM fungi in relation to several plant enzymatic activities has been often reported (Azcón *et al.*, 1996; Azcón and Tobar 1998; Calvente, 2003). In contrast to the GR activity, the lower oxidative damage to lipids in AM plants seems to be a consistent effect of AM symbiosis, regardless of the fungal species involved in the association (Ruiz-Lozano *et al.*, 2001b; Porcel *et al.*, 2003; the present results).

In addition to the above-discussed drought-tolerance mechanisms, the AM contribution to plant drought tolerance might also have occurred through drought avoidance mechanisms such as hyphal water uptake (Hardie, 1985; Ruiz-Lozano and Azcón, 1995; Marulanda *et al.*, 2003) or increased water uptake related to mycorrhizal changes in root morphology (Kothari *et al.*, 1990) or soil structure (Augé *et al.*, 2001a). Such mycorrhizal effects could allow plants to remain more hydrated than non-AM plants as soil dries (Augé *et al.*, 2001b). Data from the present study, such as the higher mid-day leaf Ψ in AM than in non-AM plants, the lower accumulation of soluble sugar and proline in shoots of AM than in non-AM plants or the lower lipid peroxidation in AM than in non-AM plants without a correlation with enhanced antioxidant activities in such plants, suggest that possibility.

The overall data show that both root and shoot tissues are influenced by AM symbiosis by means of drought-avoidance and drought-tolerance mechanisms. It seems that first the AM symbiosis enhances osmotic adjustment in roots which could contribute to maintaining a Ψ gradient favourable to the water passing from soil into the roots. This enables higher leaf Ψ in AM plants during drought and keeps the plants protected against oxidative stress, and these cumulative effects increase the plant drought tolerance.

2.5. Acknowledgements

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EVALUACIÓN DEL PAPEL DE GENES QUE CODIFICAN DEHIDRINAS (LEA, D-11) BAJO CONDICIONES DE DÉFICIT HÍDRICO EN PLANTAS MICORRIZADAS DE *Glycine max* Y *Lactuca sativa*

Resumen

En este estudio se ha determinado si la simbiosis micorricico arbuscular (MA) es capaz de alterar el patrón de acumulación de transcritos de dehidrina (LEA, grupo D-11) bajo condiciones de déficit hídrico, y si esta posible alteración está relacionada con la protección de las plantas hospedadoras frente a la sequía. Se han clonado dos genes que codifican dehidrinas de *Glycine max* (*gmlea 8* y *gmlea 10*) y uno de *Lactuca sativa* (*lslea 1*). Se ha analizado su contribución a la respuesta frente a la sequía en plantas micorrizadas de soja y lechuga. Los resultados con las plantas de soja mostraron que la mayoría de los tratamientos no presentaron expresión del gen LEA cuando se cultivaron en condiciones óptimas de riego. La mayor expresión se encontró en las plantas control no inoculadas sometidas a sequía. Sólo las plantas inoculadas únicamente con *Bradyrhizobium japonicum* mostraron un significativo nivel de expresión del gen LEA bajo condiciones óptimas y un nivel inferior de expresión bajo condiciones de estrés hídrico. Los mismos resultados se confirmaron en posteriores experimentos y en la última fase de un experimento de evolución temporal de la expresión. En lechuga, el gen *lslea 1* también fue inducido por estrés hídrico en todos los tratamientos. Sin embargo, el nivel de inducción fue claramente mayor en raíces de plantas no inoculadas que en raíces de los dos tratamientos MA ensayados. La totalidad de los resultados demostró que los niveles de acumulación de transcritos en los tratamientos micorrizados sometidos a sequía fueron considerablemente más bajos que en las correspondientes plantas no micorrizadas, indicando que la acumulación de proteínas LEA no es un mecanismo por el cual la simbiosis MA proteja a las plantas hospedadoras frente a la sequía.

Palabras clave: simbiosis micorrícico arbuscular, déficit hídrico, dehidrina, proteína LEA

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

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 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 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, *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.

Key words: *arbuscular mycorrhizal symbiosis, dehydrin, drought stress, LEA protein.*

3.1. 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 *at 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 *et al.,* 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 universal plants 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 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.

3.2. Materials and Methods

3.2.1. Experimental designs and statistical analysis

First experiment (with Glycine max)

The experiment consisted of a randomized complete block design with six inoculation treatments: (1) plants inoculated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, strain USDA 110 (Br), (2) plants inoculated with the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *B. japonicum* (Gm+Br), (3) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) and *B. japonicum* (Gi+Br) (4) plants inoculated with *G. mosseae* (Gm), (5) plants inoculated with *G. intraradices* (Gi), and (6) non-inoculated control plants (NI). These six inoculation treatments included the different combinations of microorganisms able to establish a symbiotic association with soybean root. There were 12 replicates of each treatments, totalling 72 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 days before harvest (35 d after inoculation).

Second experiment (with Glycine max)

The experiment consisted of a randomized complete block design with three inoculation treatments: (1) plants inoculated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, strain USDA 110 (Br), (2) plants inoculated with the AM fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *B. japonicum* (Gm+Br) and (3) 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 6 replicates for plant harvested after 35 d, totalling 108 pots (one plant per pot). Half of the plants

was cultivated under well-watered conditions throughout 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 treatments: (1) plants inoculated with the AM fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (Gm), (2) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) (Gi) and (3) 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.

3.2.2. 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_2O_2 solution for 8 min, then washed several times with sterile water to remove any trace of chemical that could interfere in 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.

3.2.3. 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 using a pressure plate apparatus and applying a pressure of one third atmosphere for 48h, 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 additional 10 d. 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 24h-period between each rewatering, the soil water content was progressively decreasing until 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 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_2HPO_4 and 2 mM NH_4NO_3 (NI plants), 0.35 mM K_2HPO_4 (Br plants), 3 mM NH_4NO_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 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.

3.2.4. 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 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.

3.2.5. Relative water content

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

3.2.6. 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).

3.2.7. 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 (EKKGIMDKIKEKLPG) segments are useful for degenerate primer design. These two stretches were used to design degenerate oligonucleotide primers as described by Numberg *et al.* (1989): primer forward 5' GTC GAC GA(GA) TAC GG(CT) AAC CC-3' and primer reverse 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 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 amplified was about 340 bp. PCR was carried out in a final volume of 50 µl containing 10mM 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 employed 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).

3.2.8. Sequencing the cloned cDNA and analysis

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).

3.2.9. 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 5X Denhardt's solution, 5X SSC, 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^{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 (Sambrook *et al.*, 1989). After washing twice for 5 min at room temperature in 2X SSC and 0.1% SDS, and twice for 15 min at 65 °C with 0.5X 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 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 two times with different set of plants.

3.2.10. Nucleotide sequence accession number

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

3.3. Results

3.3.1. Symbiotic development in soybean and lettuce plants

Non-inoculated soybean plants did not show any mycorrhizal infection or nodules from *Bradyrhizobium* (Table 1).

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

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 days.

[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).

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*.

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).

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	73 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

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 days 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.

[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).

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).

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

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 days.

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

3.3.2. 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 as compared to 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, plants colonized by *G. mosseae* had a significantly higher *RWC* than non-inoculated plants.

3.3.3. Cloning *gmlea* and *lslea* genes

The use of the degenerate primers designed on the Y and K segments of dehydrins allowed to obtain several clones 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. Such 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 = $2e^{-11}$).

3.3.4. 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 roots RNA from a variety of microbial treatments (see experimental design). The results obtained were very similar for both *gmlea* probes (Figure 1).

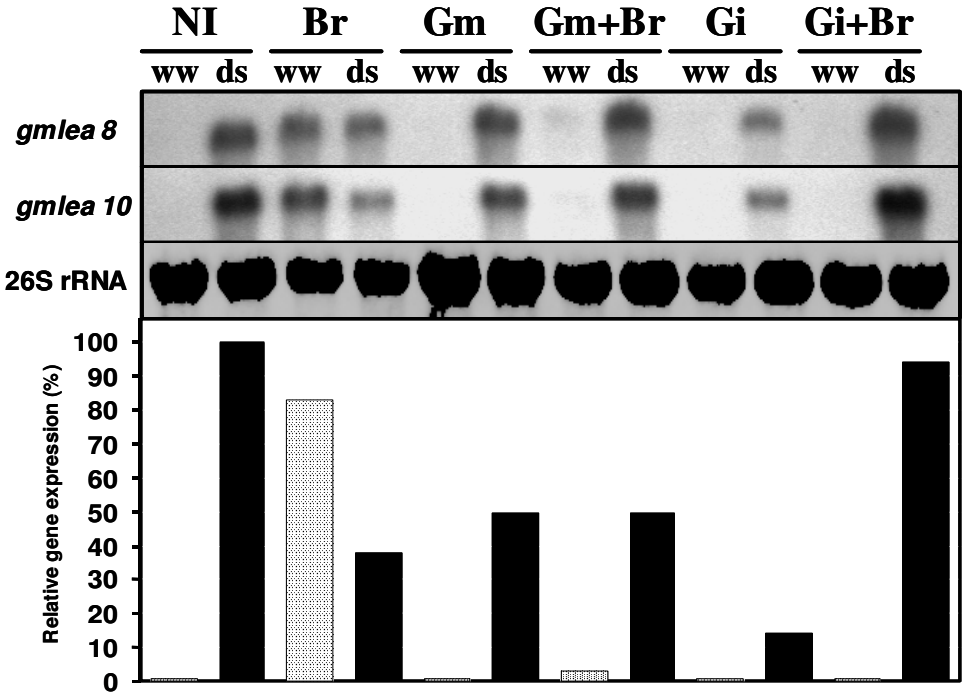

Figure 1. Northern blot of total RNA (15 µg) from soybean roots. Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Gm, *Glomus moosseae*; Gm+Br, *G. mosseae* plus *B. japonicum*; Gi, *Glomus intraradices*; Gi+Br, *G. intraradices* plus *B. japonicum*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. 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 percentage of the value for droughted non-inoculated plants

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 an important level of LEA gene expression under well-watered conditions (83% compared to 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 as compared to the other treatments, we repeated the northern blot analysis with a different set of plants (data not shown). The results obtained agreed with the previous findings since plants inoculated only with *B. japonicum* showed once more important LEA transcript accumulation under well-watered conditions; and plants inoculated with *G. intraradices* only rendered again 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* we planned a time-course experiment 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 (Figure 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 to the other two treatments (about 210% as compared to 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 (Figure 1) since plants inoculated only with *B. japonicum* showed an important expression level under well-watered conditions (about 78% as compared to non-inoculated plants) and few expression under drought conditions (9% as compared to droughted non-inoculated plants). Plants dually inoculated with *B. japonicum* plus *G. mosseae* also showed little expression under well-watered conditions (11% of non-inoculated plants), but enhanced expression under drought stress (70% of non-inoculated plants).

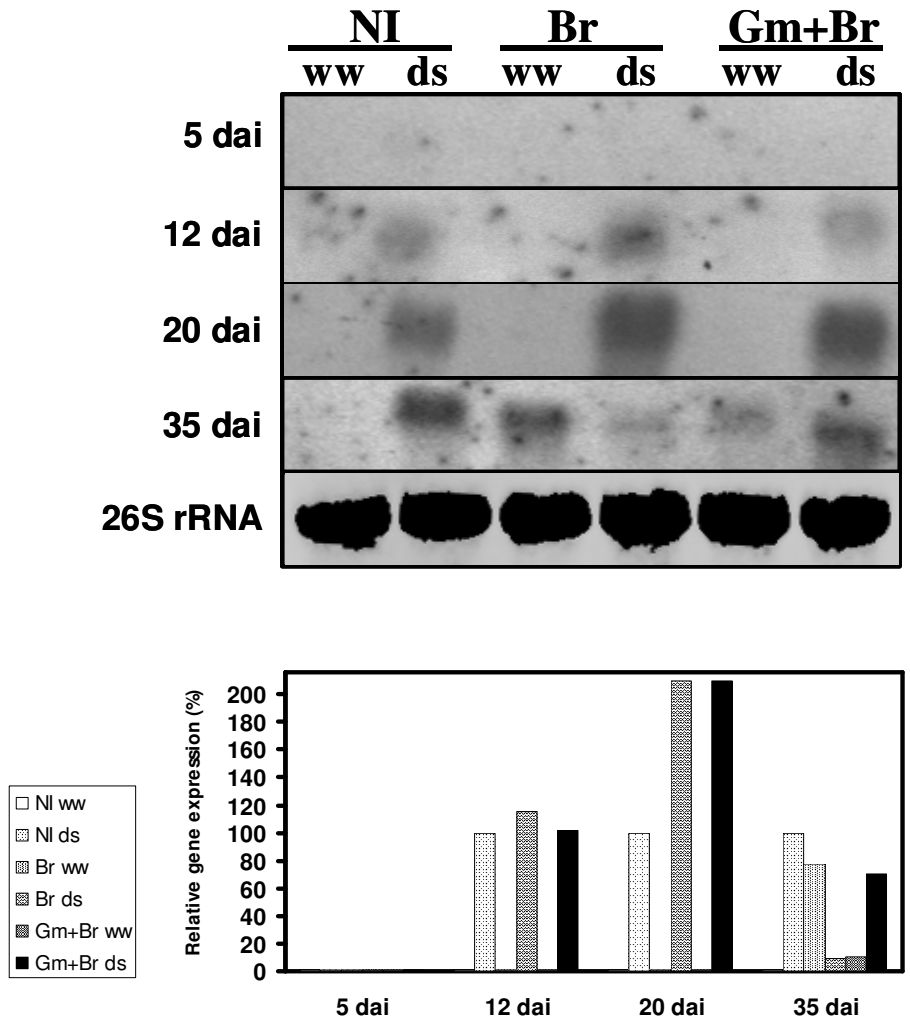

Figure 2. Northern blot of total RNA (15 µg) from soybean roots. Plants were harvested 5, 12, 20 or 35 days after inoculation (dai). Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Gm+Br, *G. mosseae* plus *B. japonicum*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. 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 percentage of the value for droughted 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 *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 microorganisms the level of LEA gene expression under drought stress conditions was lower than in the non-AM plants (45% of Br plants).

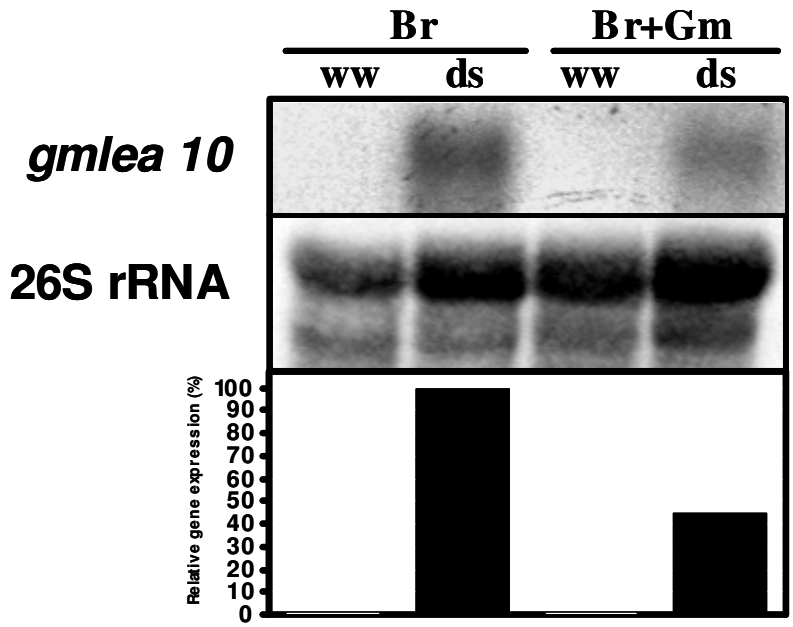

Figure 3. Northern blot of total RNA (10 µg) from soybean nodules. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm+Br, *G. mosseae* plus *B. japonicum*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The lower panel shows the amount of 26S rRNA loaded for each treatment (methylene blue staining). Histogram shows the relative gene expression (after normalization to rRNA) presented as percentage of the value for droughted non-inoculated plants.

3.3.5. 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 of AM lettuce roots that cultivated either under well-watered or drought stressed conditions. Results showed that *lslea 1* gene was also induced by drought stress in the three treatments assayed (Figure 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% as compared to non-inoculated plants, and the level of expression in plants colonized by *G. intraradices* was 64% as compared to the non-inoculated plants.

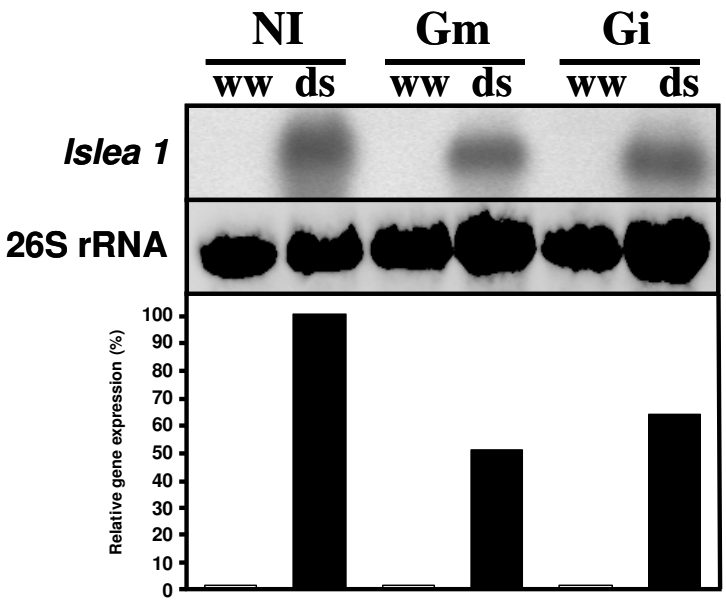

Figure 4. Northern blot of total RNA (15 µg) from lettuce roots. Treatments are designed as NI, non-inoculated controls; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*; Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The lower panel shows the amount of 26S rRNA loaded for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

3.4. 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 we have extended previous physiological observations 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 still known about how AM fungi influence plant gene expression.

The analysis of *gmlea* genes 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 only with *B. japonicum*. In nodules, the expression *of 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 be simply 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* genes expression in nodulated, drought stressed AM plants were similar to those from non-inoculated plants or to those from not-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 *gmlea genes* expression in these double inoculated plants as compared to single nodulated soybean plants.

In the time-course study we observed that at 12 and at 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 microorganisms. In fact, at 12 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 and 20 dai than at 35 dai, when both symbioses must be completely functional. In support of that, we found that the RWC of AM plants was significantly different to 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. intraradices* alone compared to that of plants colonized by *G. mosseae* alone (see Figure 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 as compared to non-inoculated plants, conversely to our initial hypothesis expecting 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 enhances first osmotic adjustment in roots, and this could contribute to maintain a water potential gradient favourable to the water entrance from 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. Also, 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, our 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 analyzed. 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.

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EVALUACIÓN DEL PAPEL DE LOS GENES QUE CODIFICAN PARA Δ^1-PIRROLIN-5-CARBOXILATO SINTETASA (P5CS) BAJO CONDICIONES DE DÉFICIT HÍDRICO EN PLANTAS MICORRIZADAS DE *Glicine max* y *Lactuca sativa*

Resumen

En este estudio se procedió a determinar si la simbiosis micorrícico arbuscular (MA) es capaz de alterar el patrón de expresión del gen Δ^1-pirrolin-5-carboxilato sintetasa (*p5cs*) bajo condiciones de déficit hídrico y si esta posible alteración se relaciona con la protección de la planta hospedadora frente a la sequía. Para llevar a cabo este objetivo, se procedió a clonar un gen que codifica para P5CS en *Glicine max* (*gmp5cs*) y otro en *Lactuca sativa* (*lsp5cs*) y se analizó su contribución a la respuesta frente la sequía en plantas de soja y lechuga control y MA. El análisis de la expresión de *gmp5cs* y *lsp5cs* mostró que estos genes estaban inducidos por estrés hídrico. La mayor expresión génica se encontró en las plantas control no inoculadas sometidas a sequía. No obstante, se obtuvo un resultado contradictorio en plantas de soja inoculadas únicamente con *Bradyrhizobium japonicum*, donde el gen *gmp5cs* mostró unos niveles de inducción muy bajos en raíces sometidas a déficit hídrico. Además, tanto en soja como en lechuga micorrizadas se observó una menor acumulación de transcritos *p5cs* bajo estrés hídrico que en las plantas control no inoculadas. Estos resultados indican indican que la inducción del gen *p5cs* no es un mecanismo por el cual la simbiosis MA proteja a la planta hospedadora frente a la sequía.

Palabras clave: Simbiosis micorrícico arbuscular, déficit hídrico, prolina, Δ^1-pirrolin-5-carboxilato sintetasa (P5CS).

EVALUATION OF THE ROLE OF GENES ENCODING FOR Δ^1-pyrroline-5-carboxylate synthetase (P5CS) DURING DROUGHT STRESS IN ARBUSCULAR MYCORRHIZAL *Glycine max* AND *Lactuca sativa* PLANTS

Abstract

In this study we have determined whether the arbuscular mycorrhizal (AM) symbiosis is able to alter the pattern of Δ^1-pyrroline-5-carboxylate synthetase (*p5cs*) gene expression under drought stress and whether such possible alteration functions in the protection of the host plants against drought. To achieve this, we cloned a P5CS-encoding gene from *Glycine max* (gm*p5cs*) and another from *Lactuca sativa* (*lsp5cs*) and analyzed their contribution to the response against drought in control and AM soybean and lettuce plants. The analysis of *gmp5cs* and *lsp5cs* gene expression showed that these genes were up-regulated by drought stress. The highest gene expression was found in non-inoculated plants subjected to drought. A contrasting result was obtained in soybean plants singly inoculated with *Bradyrhizobium japonicum*, where the *gmp5cs* gene showed little up-regulation in roots under drought stressed conditions. Moreover, both soybean and lettuce AM plants showed lower *p5cs* transcript accumulation under drought stress than non-inoculated plants. The present results indicate that the induction of *p5cs* gene is not a mechanism by which the AM symbiosis protects their host plant against drought.

Key words: *arbuscular mycorrhizal symbiosis, drought stress, proline, Δ^1-pyrroline-5-carboxylate synthetase (P5CS)*

4.1. Introduction

Plants are, in nature, frequently exposed to adverse environmental conditions that have a negative effect on plants physiology. 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 [13, 36].

The arbuscular mycorrhizal (AM) fungi are widespread microorganisms able to establish a symbiotic association with the roots of most terrestrial plants. AM plants have improved ability for nutrient uptake and tolerance to biotic and abiotic stresses while the fungus acquires a protected ecological niche and plant photosynthates [64]. The AM symbiosis is present in all natural ecosystems, even in those affected by adverse environmental conditions [7], and it can be defined

as a specialized system for nutrient uptake and transfer, more efficient than roots alone [66]. Nevertheless, the physiological role of the AM symbiosis is not limited to uptake and transfer of nutrients to the host plant. Many other beneficial effects for the host plant and for ecosystems have been described [64], including enhancement of tolerance to drought stress [3, 5, 49, 51, 53, 56]. 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 [51].

Plants, can also 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 [11]. The typical first response of all living organisms to water deficit is osmotic adjustment. To counter with drought stress, many plants increase the osmotic potential of their cells by synthesizing and accumulating compatible osmolytes such as proline that participates in the osmotic adjustment [32, 35, 42]. However, proline performs also an important function as a protective compatible osmolyte in scavenging of free radicals and facilitating a correction of altered redox potential by replenishment of the NADP+ supply [23, 26]. Accumulation of proline is due primarily to *de novo* synthesis, although a reduced rate of catabolism has also been observed [35]. The first two steps of proline biosynthesis are catalized by Δ^1-pyrroline-5-carboxylate synthetase (P5CS) by means of its γ-glutamil kinase and glutamic-γ-semialdehyde dehydrogenase activities. Subsequently, the Δ^1-pyrroline-5-carboxylate (P5C) formed is reduced by P5C reductase (P5CR) to proline [32]. The rate-limiting step in this pathway is represented by the γ-glutamil kinase activity of P5CS, which is sensitive to feedback inhibition by relatively low levels of proline [63]. In addition, in *Arabidopsis*, the P5CS-encoding gene is induced by drought stress, salinity and ABA, but P5CR is not [68]. The overexpression of the P5CS-encoding gene in transgenic tobacco plants has been shown to increase proline production and to confer tolerance of such plants to osmotic stress [35]. Hence, the P5CS-encoding gene is of key importance for the biosynthesis of proline in plants [1].

Investigations carried out so far on osmorregulation in the AM symbiosis are scarce and somewhat contradictory. While some studies have shown an increase in proline accumulation in mycorrhizal plants subjected to drought [6, 22, 53], the same studies also demonstrated that the increase in proline accumulation was quite variable depending on the AM fungus involved. For instance, while plants colonized by *G. deserticola* accumulated 120 nmol of proline per g fresh weight, plants colonized by *G. intraradices* only accumulated 41 nmol proline per g fresh weight [53]. It has also been shown that under low Ca in the medium AM plants accumulated more proline than non-AM plants when subjected to PEG-induced drought stress, while under high Ca in the medium this was not so [52]. On the contrary, other studies regarding drought [50] or salt stress [54] have shown a lower proline accumulation in AM plants than in non-AM ones.

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 [51]. Moreover, a molecular basis for the tolerance to water stress in AM plants remains far from being understood. The establishment of the expression pattern of genes such as *p5cs* in AM plants under osmotic stress conditions, should provide an insight into the role of the AM symbiosis in the process of osmotic adjustment during drought stress. As a first approach, in the present study we have cloned a P5CS-encoding gene from *Glycine max* (*gmp5cs*) and another from *Lactuca sativa* (*lsp5cs*) and analyzed their contribution to the response against drought in mycorrhizal soybean and lettuce plants.

4.2. Materials and Methods

4.2.1. Experimental designs

First experiment (with Glycine max)

When considering experiments with legume plants the control plants are not only the non-inoculated plants, but also the plant singly inoculated with the corresponding *Rhizobium*. For that reason this experiment was designed to cover the different inoculation combinations of *Bradyrhizobium japonicum* and the two AM fungi used. The experiment consisted of six inoculation treatments: (1) plants inoculated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, strain USDA 110 (Br), (2) plants inoculated with the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *B. japonicum* (Gm+Br), (3) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) and *B. japonicum* (Gi+Br) (4) plants inoculated with *G. mosseae* (Gm), (5) plants inoculated with *G. intraradices* (Gi), and (6) non-inoculated control plants (NI). Twelve replicates of each treatment were done totalling 72 pots (one plant per pot) so that half of them were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 10 days before harvest.

Second experiment (with Glycine max)

The experiment consisted of a randomized complete block design with three inoculation treatments: (1) plants inoculated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, strain USDA 110 (Br), (2) plants inoculated with the AM fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *B. japonicum* (Gm+Br) and (3) non-inoculated control plants (NI). For each treatment plants were cultivated at four time intervals: 5, 12, 20 or 35 days after inoculation (dai). A variable number of replicates of each treatment was done, ranging from 12 for plants to be harvested after only 5 dai to 6 for plant to be harvested after 35 dai, totalling 108 pots (one plant per pot) so that half of them were cultivated under well-watered conditions throughout the entire experiment,

while the other half were drought-stressed for 5 days (for plants harvested 5 dai) or for 10 days (for the rest of treatments) before harvest.

Third experiment (with Lactuca sativa)

The experiment consisted of a randomized complete block design with three inoculation treatments: (1) plants inoculated with the AM fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (Gm), (2) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) (Gi) and (3) non-inoculated control plants (NI). Ten replicates of each treatment were done totalling 30 pots (one plant per pot) so that half of them were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 10 days before harvest.

Fourth experiment (with Lactuca sativa)

The experiment consisted of a randomized complete block design with two inoculation treatments: (1) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) (Gi) and (2) non-inoculated control plants (NI) and two rates of proline added to the medium: (1) 10 mM proline (+Pro) or no proline added (-Pro). Ten replicates of each treatment were done totalling 80 pots (one plant per pot) so that half of them were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 10 days before harvest.

4.2.2. 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 days). 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_2O_2 solution for 8 min, then washed several times with sterile water to remove any trace of chemical that could interfere in 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.

4.2.3. 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 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 experiment). At this time half of the plants were allowed to dry until soil water content reached 70% field capacity (two 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 10 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 24h-period comprised between each rewatering the soil water content was progressively decreasing until 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 days (plants harvested 5 dai) or for 10 days for the rest of harvests. In the third and fourth experiments plants were maintained at field capacity during 5 weeks and then half of the plants was subjected to drought stress (70% of field capacity) for two weeks, while the other half remained under well-watered conditions.

Each week throughout the experiment, soybean plants received 10 ml of Hewitt´s nutrient solution lacking N and P [29]. Three weeks after planting, plants received nutrient solution amended with N and/or P as follows [21]: 0.18 mM K_2HPO_4 and 2 mM NH_4NO_3 (NI plants), 0.35 mM K_2HPO_4 (Br plants), 3 mM

NH_4NO_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 [29], modified to contain 4 mM N + 1 mM P. Mycorrhizal plants did not receive nutrient solution. The use of such fertilization level for non-mycorrhizal plants was meant to obtain well-watered control plants of similar size and nutrient contents to the AM plants tested in this assay.

In the second experiment with lettuce, proline was added as an aqueous solution at a rate of 30 ml per week, so that at the end of the experiment the total amount of proline added was 10 mM per pot.

4.2.4. 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 & Hayman [47]. The extent of mycorrhizal colonization was calculated according to the gridline intersect method [20]. Nodule number in soybean roots was determined using a binocular microscope.

4.2.5. Proline content

Free proline was extracted from 0.3 g of fresh tissue and determined by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates et al. [8].

4.2.6. RNA isolation and synthesis of first strand cDNA

Total RNA was isolated from soybean or lettuce roots by phenol/chloroform extraction [34]. 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 fwt) that had been previously separated from the roots at the harvest time and kept at –80 °C in order to be used for other biochemical determinations. The RNA from nodules was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

4.2.7. Cloning the *gmp5cs* and *lsp5cs* genes

Several stretches of conserved amino acids were apparent from the compilations of sequences for the P5CS protein in plants. Two stretches were

used to design degenerate oligonucleotide primers as described by Numberg et al. [43]: primer forward 5'-GGT GT(TA) CTC CTG AT(TG) GT(TC) TTT GA-3' and primer reverse 5'-CC (TC)TC AAC (TC)CC (TG)AC (TA)GG (AT)CC-3'. Using cDNA from either soybean or from lettuce roots subjected to drought stress as template, a cDNA fragment of about 840 bp was amplified with these primers and the polymerase chain reaction (PCR). PCR was carried out in a final volume of 50 µl containing 10mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 100 pmoles of each primer and 2 units of *Taq* DNA polymerase (Sigma, St. Louis, MO, USA). A Perkin/Elmer thermocycler (model 2400) was employed with the following values: initial denaturation at 95 °C for 5 min, followed by 35 denaturation cycles at 95 °C for 45 sec, annealing at 52 °C for 45 sec, elongation at 72 °C for 75 sec, 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 pGEM 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).

4.2.8. Sequencing the cloned cDNA and analysis

Sequencing was performed by the dideoxy-sequencing method [57] 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 Centre for Biotechnology Information (NCBI).

4.2.9. 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 [55]. Equal RNA loading and transfer were verified by methylene blue staining of nylon membranes before hydridization [28]. Blots were prehybridized 2-3 h at 42 °C in 5X Denhardt's solution, 5X SSC, 0.5% SDS and hybridized with gmp5cs or lsp5cs specific probes obtained by radioactive PCR labelling of plasmid inserts. 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 [55]. After washing twice for 5 min at room temperature in 2X SSC and 0.1% SDS, and twice for 15 min at 65°C with 0.5X 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 (BioRad, 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.

4.2.10. Nucleotide sequence accession number

The nucleotide sequences corresponding to *gmp5cs* and *lsp5cs* cDNAs have been deposited in the EMBL database under accession numbers AJ715851, and AJ715852 respectively.

4.2.11. Statistical analysis

Data on proline content and root fresh weight were subjected to analysis of variance (ANOVA) with microbial treatment, water regime and microbial treatment-water regime interaction as sources of variation, and followed by Duncan´s multiple range test [18].

4.3. Results

4.3.1. Symbiotic development in soybean and lettuce plants

No AM colonization or nodules were observed in non-inoculated soybean plants. The percentage of AM infection in plants from the first experiment ranged from 86% to 92% and the number of nodules in *B. japonicum*-inoculated plants from 30 to 50 (data not shown). In the time-course experiment, mycorrhizal infection inside roots and nodule formation were visible 12 dai and both symbioses were progressing until the last harvest (35 dai). The AM colonization at 12 dai was 17% (ww) and 15% (ds) of mycorrhizal root length, at 20 dai it reached 30% (ww) and 25% (ds) and at 35 dai it was 55% (ww) and 47(ds), while the number of nodules ranged from 20 to 30 in *B. japonicum*-inoculated plants (data not shown).

The percentage of AM colonization was about 80% in both lettuce experiments and resulted unaffected by drought stress and by proline addition (data not shown).

4.3.2. Root development

The root fresh weight of soybean plants (experiment 1) is shown in Figure 1. Drought stress reduced root fresh weight in all treatments. Plants dually inoculated with *G. intraradices* plus *B. japonicum* showed the highest root development under drought stress conditions, while non-inoculated plants showed the lowest root development.

The root fresh weight of lettuce plants (experiment 1) also was reduced by drought stress in all treatments (Figure 2). However, the reduction in root development was lower in both AM treatments than in the non-AM plants.

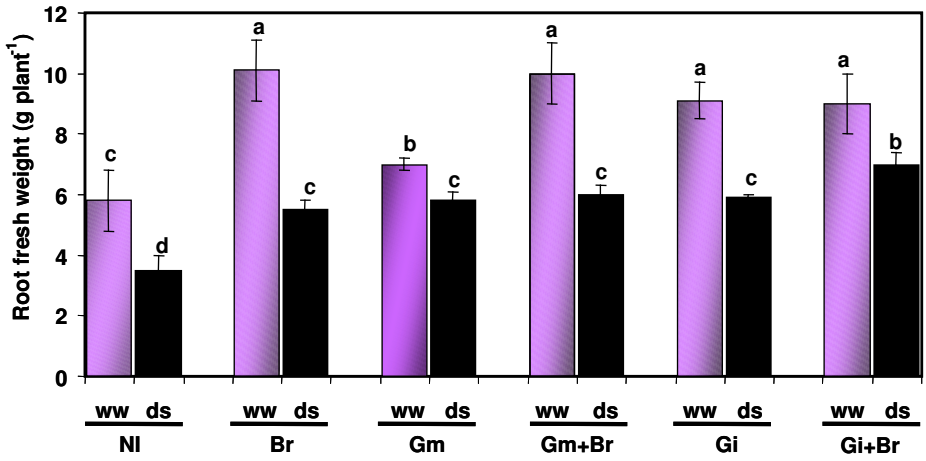

Figure 1. Root fresh weight of soybean plants. Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Gm, *Glomus mosseae*; Gm+Br, *G. mosseae* plus *B. japonicum*; Gi, *Glomus intraradices*; Gi+Br, *G. intraradices* plus *B. japonicum*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days.

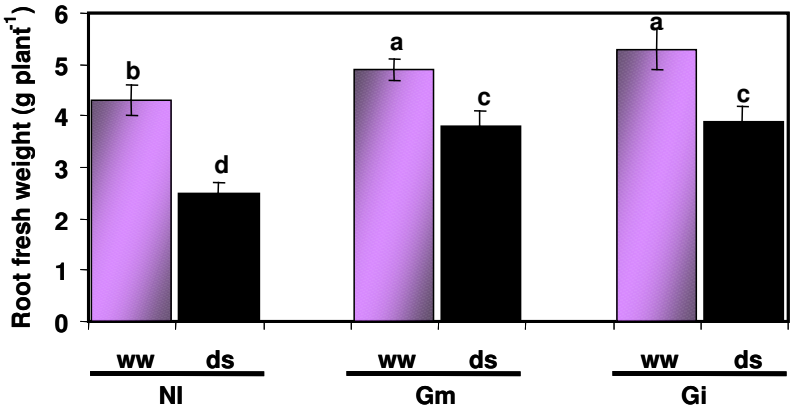

Figure 2. Root fresh weight of lettuce plants. Treatments are designed as NI, non-inoculated controls; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*; Plants were either well-watered (ww) or drought stressed (ds) for 10 days.

4.3.3. Cloning *gmp5cs* and *lsp5cs* genes

The use of the degenerate primers designed on conserved segments of P5CS allowed to obtain several clones containing inserts of the expected size using cDNA from soybean and from lettuce roots subjected to drought stress. The sequencing of several of the clones obtained from soybean cDNA showed that one of them contained a cDNA insert putatively encoding for a P5CS protein. The clone was named *gmp5cs*. Such clone contained a cDNA fragment of 831 bp and the putative protein encoded gave 76% identity with a P5CS protein from *Vigna unguiculata* (accession Q9AYM4, e = $1e^{-114}$).

In the case of lettuce, we also sequenced several clones and only one of them contained a cDNA insert putatively encoding for a P5CS protein. Such clone was named *lsp5cs*. It contained a cDNA fragment of 839 bp. The putative protein encoded by that cDNA gave 72% identity with the P5CS from *Mesembryanthemum crystallinum* (accession O65361, e = $1e^{-113}$).

4.3.4. Northern blot analysis with soybean RNAs and proline content

The cDNA insert from soybean (*gmp5cs*) was used as probe in northern blot analyses with RNA from soybean roots from a variety of microbial treatments (see experimental design). The results obtained are shown in Figure 3. The higher gene expression was found in non-inoculated plants subjected to drought that was set as 100% in arbitrary units after normalization of northern according to the amount of ribosomal RNA loaded in the blots. Under well-watered conditions, all the treatments showed slight but constitutive gene expression, which ranged from 1.8% for plants singly inoculated with *B. japonicum* to 0.1% for plants singly inoculated with *G. mosseae* or with *G. intraradices*. Drought stress up-regulated the level of *gmp5cs* transcript accumulation in all treatments. Only plants singly inoculated with *B. japonicum* showed a reduced level of up-regulation in *gmp5cs* gene expression under drought stress conditions (only 12% of non-inoculated plants). Plants singly inoculated with *G. mosseae* showed up-regulation of *gmp5cs* gene expression under drought stress (47% of the level found in non-inoculated plants), and a similar level of gene expression was found in those dually inoculated with *B. japonicum* plus *G. mosseae* under drought stress conditions (51% of non-inoculated plants). Plants singly inoculated with *G. intraradices* only showed detectable *gmp5cs* gene expression under drought stress conditions, but this expression was lower than in the other fungal treatment (23% of non-inoculated plants). Finally, plants dually inoculated with *B. japonicum* plus *G. intraradices* also exhibited *gmp5cs* gene expression under drought stress conditions (50% of non-inoculated plants).

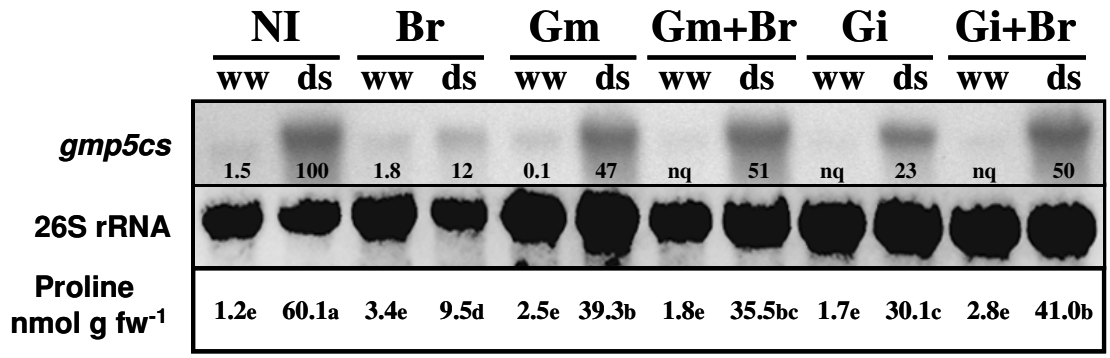

Figure 3. Northern blot of total RNA (15 µg) from soybean roots. Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Gm, *Glomus mosseae*; Gm+Br, *G. mosseae* plus *B. japonicum*; Gi, *Glomus intraradices*; Gi+Br, *G. intraradices* plus *B. japonicum*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The percentage of gene expression is indicated by numbers close to each northern (nq means "not quantifiable"). The second panel shows the amount of 26S rRNA loaded for each treatment (methylene blue staining). The third panel shows the proline content in these roots.

The proline content was also maximum in non-inoculated plants subjected to drought stress (Figure 3), followed by all the AM plants subjected to drought. However, plans singly inoculated with *G. intraradices* or with *B. japonicum* accumulated less proline under drought stress than the rest of treatments (as

also happened with the *gmp5cs* transcripts). The level of proline accumulated in roots from well-watered plants was low in all treatments.

RNA from soybean root nodules was also extracted and used for northern blot analysis of *p5cs* transcript accumulation. Figure 4 shows results obtained with *gmp5cs* probe in nodules from plants inoculated only with *B. japonicum* or dually inoculated with *B. japonicum* plus *G. mosseae* (the fungus that had showed a more normal behaviour in relation to gm*p5cs* gene expression as compared to *G. intraradices*, see Figure 3).

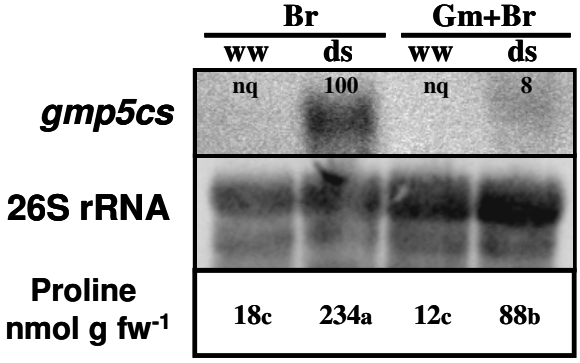

Figure 4. Northern blot of total RNA (10 µg) from soybean nodules. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm+Br, *G. mosseae* plus *B. japonicum*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The percentage of gene expression is indicated by numbers close to each northern (nq means "not quantifiable"). The second panel shows the amount of 26S rRNA loaded for each treatment (methylene blue staining). The third panel shows the proline content in these nodules.

It is clearly visible that in nodules of *B. japonicum*-inoculated plants there was no expression of gm*p5cs* gene in well-watered plants, while the expression increased notably in nodules of these plants when subjected to drought stress (which was set as 100%). In nodules from plants dually inoculated with both symbiotic microorganisms the level of *gmp5cs* gene expression under drought stress conditions was lower than in the nonAM plants (8% of Br plants).

Proline also accumulated considerably more in nodules of non-AM plants subjected to drought than in the corresponding AM plants. Following the low up-regulation of *gmp5cs* gene expression and low proline accumulation in roots from plants singly inoculated with *B. japonicum* and subjected to drought, we planed a time-course experiment in order to study the expression level of *gmp5cs* gene at different time intervals in non-inoculated soybean plants or after inoculation with either *B. japonicum* or with *B. japonicum* plus *G. mosseae* (the fungus that had showed a more normal behaviour in relation to *gmp5cs* gene expression as compared to *G. intraradices*, see Figure 3). All plants harvested at 5 dai showed a low but constitutive level of gm*p5cs* transcript accumulation under whatever conditions (Figure 5).

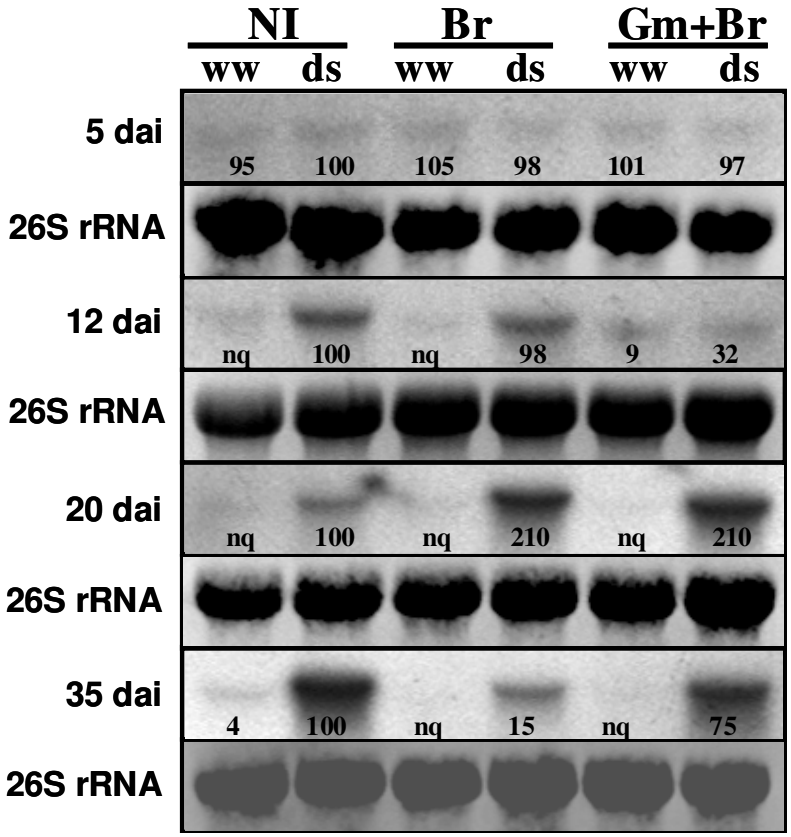

Figure 5. Northern blot of total RNA (15 µg) from soybean roots with *gmp5cs*. Plants were harvested 5, 12, 20 or 35 days after inoculation (dai). Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Gm+Br, *G. mosseae* plus *B. japonicum*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The percentage of gene expression is indicated by numbers close to each northern (nq means "not quantifiable"). The panel under each northern shows the amount of 26S rRNA loaded for each treatment (methylene blue staining).

At 12 dai, *gmp5cs* transcript accumulation was up-regulated in the three treatments when subjected to drought. However, plants dually inoculated with *B. japonicum* plus *G. mosseae*, showed a considerably lower up-regulation of gene expression. After normalization, the level of gene expression in arbitrary units was 100% for non-inoculated plants, 98% for *B. japonicum*-inoculated plants and 32% for dually inoculated plants. At 20 dai, again *gmp5cs* transcript accumulated in the tree treatments subjected to drought, but, surprisingly, the level of expression was considerably lower in the non-inoculated plants (set as 100%) than in the other two treatments (about 210% of non-inoculated plants). Finally at 35 dai, the patter of *gmp5cs* transcript accumulation returned similar to that observed in plants from the first experiment (Figure 3) since non-inoculated plants exhibited the highest levels of transcript accumulation under drought stress (set as 100%), while plants inoculated only with *B. japonicum* showed little *gmp5cs* gene expression under drought conditions (15% of non-inoculated plants). Plants dually inoculated with *B. japonicum* plus *G. mosseae* also showed enhanced

gene expression under drought stress, but lower than in non-inoculated plants (75% of non-inoculated plants).

4.3.5. Northern blot analysis with lettuce RNA and proline content

In order to test the behaviour of the P5CS gene in a non legume plant and avoid the interference of AM symbiosis with that of *Bradyrhizobium* symbiosis, the cDNA clone from lettuce (*lsp5cs*) was also used for northern blot analysis with RNA from non-inoculated of AM lettuce roots cultivated under well-watered or drought stressed conditions.

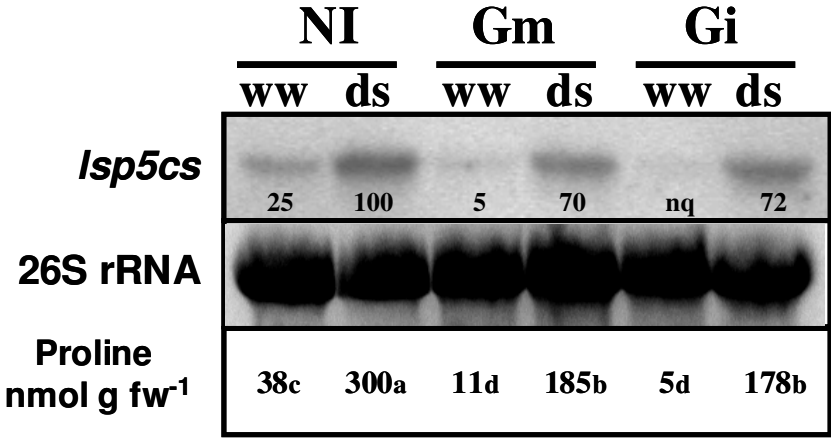

Figure 6. Northern blot of total RNA (15 µg) from lettuce roots. Treatments are designed as NI, non-inoculated controls; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*; Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The percentage of gene expression is indicated by numbers close to each northern (nq means "not quantifiable"). The second panel shows the amount of 26S rRNA loaded for each treatment (methylene blue staining). The third panel shows the proline content in these roots.

Results (Figure 6) showed that under well-watered conditions there was a low but constitutive *lsp5cs* gene expression the three treatments assayed (more visible in non-inoculated plants). Drought stress induced *lsp5cs* transcript accumulation in the three treatments. However, the level of induction was 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 *lsp5cs* gene expression in non-inoculated plants was set as 100%, the level of *lsp5cs* gene expression in plants colonized by *G. mosseae* was 70% as compared to non-inoculated plants, and the level of expression in plants colonized by *G. intraradices* was 72% as compared to the non-inoculated plants.

The highest accumulation of proline was observed in non-inoculated plants subjected to drought stress, followed by both AM stressed treatments (Figure 6). Non-inoculated plants accumulated slightly higher levels of proline under well-watered conditions than well-watered AM plants.

We planed a second experiment with lettuce plants added or not of proline (the osmolyte in whose biosynthesis participates the P5CS). In that case, since both AM fungi tested affected in a similar way the expression of *lsp5cs* gene, we

used *G. intraradices* as the colonizing AM fungus because in previous experiments with lettuce plants it has shown to be very effective protecting the host plants against drought [41]. The expression of *lsp5cs* genes was analyzed in roots of AM or control lettuce plants added or not of 10 mM proline during the growth period. Results obtained (Figure 7) showed that in root tissues of non-inoculated plants *lsp5cs* transcripts accumulated both under well-watered and under drought stressed conditions (regardless of proline addition), while in *G. intraradices*-colonized plants, the expression was only detected under drought stressed conditions (regardless of proline addition).

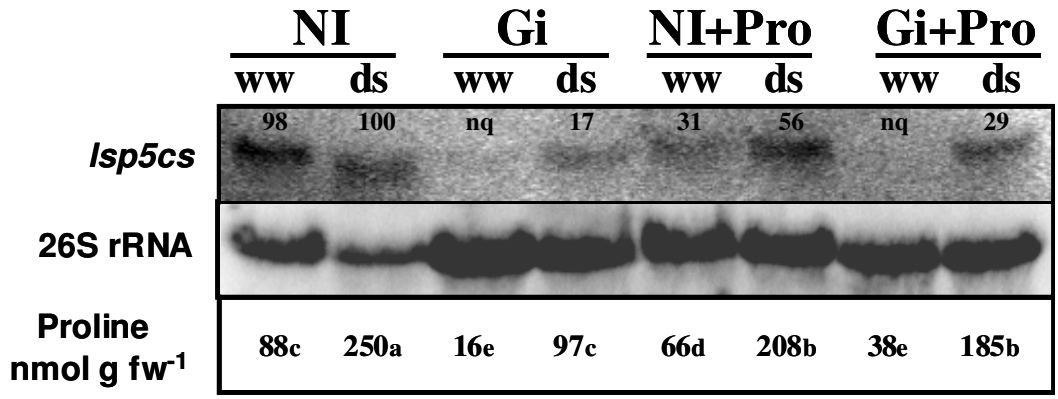

Figure 7. Northern blot of total RNA (15 µg) from lettuce roots added or not of 10mM proline (+Pro). Treatments are designed as NI, non-inoculated controls; Gi, *Glomus intraradices*; Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The percentage of gene expression is indicated by numbers close to each northern (nq means "not quantifiable"). The second panel shows the amount of 26S rRNA loaded for each treatment (methylene blue staining). The third panel shows the proline content in these roots.

The highest expression of *lsp5cs* gene was found in roots from non-inoculated plants without proline addition, both under well-watered and under drought stress conditions. The two values of non-inoculated plants, after normalization according to the RNA loaded in the membrane, were set up as 98% and 100%, respectively. Plants colonized with *G. intraradices* and not supplied with proline only showed 17% of *lsp5cs* transcript accumulation under drought stress conditions. The addition of proline to non-inoculated plants decreased the level of *lsp5cs* transcript accumulation to 31% under well-watered conditions and to 56% under drought stress conditions. The addition of proline to plants colonized by *G. intraradices* slightly enhanced the accumulation of *lsp5cs* transcript until 29% as compared to the same plants not supplied with proline.

Non-inoculated plants subjected to drought and not supplied with proline accumulated the highest amount of proline (Figure 7). The two plant treatments added of proline and subjected to drought also accumulated important amounts of proline. Curiously, the non-inoculated plants cultivated under well-watered conditions also showed significant levels of proline and accumulated a similar amount of proline as the stressed AM plants not supplied with proline.

4.4. Discussion

As a soil dries out and its water potential becomes more negative, plants must decrease their water potential to maintain a favourable water flow gradient from soil into roots. The most important mechanism to achieve such an effect, known as osmotic adjustment or osmoregulation, is to decrease the plant osmotic potential by active accumulation of organic ions or solutes [30, 42]. Of these metabolites, proline is probably the most widespread in plants [2, 44, 65, 69]. 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 osmorregulation and osmotolerance [15]. Moreover, it has been shown to protect enzymes from inactivation by salinity, heat, chilling and dilution *in vitro* [16, 59].

In previous studies we have shown several physiological and biochemical mechanisms by which the AM symbiosis protected the host plants against the detrimental effects of drought [51]. In contrast, molecular investigations in AM symbiosis have been far less common to date than physiological studies. In this study we have extended previous physiological observations by studying at the molecular level the possible participation of genes encoding for P5CS, the enzyme catalyzing the rate-limiting step in proline biosynthesis [2, 35, 71], in the enhanced tolerance to drought stress in mycorrhizal soybean and lettuce plants. In fact, several investigations on the relationship between the expression of the key gene involved in the synthesis of proline and the accumulation of proline under water stress indicate that the level of proline in plants is mainly regulated at the transcriptional level during water stress [1, 2, 14, 32, 68, 69, 70].

The analysis of *gmp5cs* and *lsp5cs* gene expression showed that, in general, these genes responded to drought and were up-regulated in drought stressed treatments, suggesting that they are important for the plant response against stresses involving water deficit [24, 35, 46, 69]. A contrasting result was obtained, however, in soybean plants singly inoculated with *B. japonicum*, where the *gmp5cs* gene showed little up-regulation in roots under drought stressed conditions. This result was latter confirmed in the time-course experiment at 35 dai. Hence, a question raises; why the level of *gmp5cs* gene expression did not increase at these plant stages in nodulated non-AM plants after drought stress?. To answer this question it must be considered that drought stress also induces synthesis and accumulation of abscisic acid (ABA), which mediates the plant responses to water deficiency, mostly by promoting stomatal closure [25], but also by inducing the expression of water deficit-responsive genes such as *p5cs* [1, 10, 11, 12, 62]. In addition, it has been demonstrated that the expression of *p5cs* genes has two regulatory pathways, an ABA-dependent and an ABA-independent pathway, and that both can act simultaneously [1, 19, 58, 61, 70]. Hence, a possible answer to the above question is that nodulation itself can be affecting one of these regulatory pathways for P5CS accumulation (i.e. the ABA-independent pathway), avoiding the accumulation of *p5cs* transcripts. In contrast, the mycorrhization of nodulated plants restore, at least in part, the normal *p5cs* transcripts accumulation pattern by compensating in some way such ABA-independent pathway.

In nodules of plants singly inoculated with *B. japonicum*, the pattern of *gmp5cs* gene expression was the expected one, namely up-regulation under drought stress conditions. An elevated rate of proline biosynthesis in nodules has been suggested to stimulate ureide synthesis in legumes and to help transfer redox potential from the nodule cytoplasm to the bacteroids [37]. Our results also showed that the amount of proline accumulated in nodules was higher than in soybean roots. Proline may also act as a carbon and nitrogen source for the bacteroids. An additional an important role of proline in nodules may be its involvement in osmoregulation [14]. In fact, the osmoticum in infected nodule cells is known to be 4- to 5-fold higher than in root cells [67]. Hence, the up-regulation of *gmp5cs* in nodules of droughted plants may represent an osmoregulatory adaptation to increased concentration of solutes. What is not explained by that hypothesis is why the expression of the *gmp5cs* gene in nodules from soybean plants dually inoculated with the *G. mosseae* and with *B. japonicum* was considerably lower than in the corresponding non-mycorrhizal plants. However, it has been proposed that mycorrhization can 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 [17, 21, 39]. Hence, an ABA-dependent regulation pathway could explain the decrease in *gmp5cs gene* expression in nodules of these double inoculated plants as compared to those of single nodulated soybean plants.

The last mechanism may explain, in the same way, why the levels of *gmp5cs* and *lsp5cs* gene expression are lower in roots from droughted soybean and lettuce AM plants than in roots from droughted soybean and lettuce non-inoculated plants.

The regulation of gene expression by ABA or water stress varies depending on the tissue or on the developmental stage of plant [33, 60]. Furthermore, *p5cs* genes can be also under developmental regulation [2, 70]. These findings can explain the little *gmp5cs* gene expression during the first stages of the time-course experiment and why the gene expression increased in subsequent stages of the time-course.

Proline has been demonstrated to ameliorate dehydration-induced perturbation in proteins [45], and exogenously supplied proline confers osmotic tolerance to the plants and cultured cells [44]. In this study, the addition of proline to lettuce plants reduced the expression of *lsp5cs* gene in all cases, except in *G. intraradices*-inoculated plants subjected to drought. It is known that P5CS protein (and, probably, *p5cs* gene expression) is feedback inhibited by proline [63]. Such feedback inhibition of P5CS by proline is lost under drought stress due to conformational changes in the P5CS protein [9, 27, 31, 40, 71]. However, it has been proposed that in some circumstances the feedback inhibition of P5CS protein (and we assume that also of *p5cs* gene, as mentioned above) is not completely eliminated under drought stress conditions [31], which could explain that *lsp5cs* gene did not increase significantly the transcript accumulation levels after addition of proline in non-inoculated or in *G. intraradices*-inoculated plants.

In any case, a consistent effect observed both in soybean and in lettuce plants is that, contrarily to our initial hypothesis expecting a possible increase of

p5cs gene expression in AM plants and contributing to the alleviation of drought stress by the AM symbiosis, the expression of *gmp5cs* and *lslp5cs* genes decreased in drought stressed AM plants as compared to non-inoculated plants. As mentioned above, this effect can be related to an hormonal or other developmental change induced in AM plants, but it is also possible that AM plants were simply less strained by drought stress and by that reason the level of *p5cs* genes expression was lower in AM than in non-AM plants. In previous studies in which we and other authors have found physiological or biochemical mechanisms involved in the enhanced tolerance to drought stress in AM plants it has also 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 [38] or soil structure [4, 5] might have contributed to the AM protection of host plants against drought [48]. Also, a recent study has revealed that, apart from direct hyphal water uptake, it seems that first the AM symbiosis enhances osmotic adjustment in roots, that can contribute to maintain a water potential gradient favourable to the water entrance from soil into the roots. This enables higher leaf water potential in AM plants during drought and keeps the plants protected against oxidative stress, and all these cumulative effects increases the plant tolerance to drought [49].

In conclusion, our results demonstrate that the cloned *p5cs* genes responded clearly to drought stress and were up-regulated under drought conditions in soybean and 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 *p5cs* genes analyzed. Moreover, the levels of *p5cs* transcript accumulation in mycorrhizal treatments subjected to drought were considerably lower than in the corresponding non-mycorrhizal plants, indicating that the induction of *p5cs* gene is not a mechanism by which the AM symbiosis protects their host plant, and suggesting that AM plants were less strained by drought than non-AM plants due to primary drought-avoidance mechanisms.

4.5. Acknowledgements

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LA DISMINUCIÓN DE LA EXPRESIÓN DEL GEN *NTAQP1* EN PLANTAS DE TABACO NO AFECTA AL PATRÓN DE COLONIZACIÓN RADICAL POR HONGOS MICORRÍCICO ARBUSCULARES PERO DISMINUYE SU EFICIENCIA SIMBIÓTICA EN CONDICIONES DE SEQUÍA

Resumen

En este trabajo, investigamos en dos líneas de tabaco (*Nicotiana tabacum*) (salvaje o mutante antisentido) si la disminución en la expresión del gen NTAQP1, que codifica para una aquaporina de membrana plasmática afecta al patrón de colonización micorrícico arbuscular (MA) o a la eficiencia simbiótica de hongos MA. Los dos objetivos se estudiaron bajo condiciones óptimas y condiciones de déficit hídrico. Ambas líneas de plantas tuvieron un patrón de colonización radical similar bajo condiciones óptimas y de déficit hídrico. Por el contrario, bajo condiciones de estrés, las plantas MA de fenotipo salvaje crecieron más rápidamente que las plantas MA antisentido. El intercambio gaseoso de la planta también dependió de la expresión del gen *NtAQP1* y fue paralelo a los incrementos en el crecimiento. Se discute más en detalle las implicaciones que tiene la mejora del transporte de agua vía simplasto a través de NtAQP1 para la eficiencia de la simbiosis MA bajo condiciones de déficit hídrico.

Palabras clave: Aquaporina, Mutante antisentido, Micorriza arbuscular, Sequía, Eficiencia simbiótica.

IMPAIRMENT OF *NtAQP1* GENE EXPRESSION IN TOBACCO PLANTS DOES NOT AFFECT ROOT COLONISATION PATTERN BY ARBUSCULAR MYCORRHIZAL FUNGI BUT DECREASES THEIR SYMBIOTIC EFFICIENCY UNDER DROUGHT

Abstract

We investigated in two tobacco (*Nicotiana tabacum*) plant lines (wildtype or antisense mutant) whether the impairment in the plasma membrane aquaporin (*NtAQP1*) gene expression affects the arbuscular mycorrhizal (AM) fungal colonization pattern or the symbiotic efficiency of AM fungi. The two objectives were investigated under well-watered and drought stress conditions. Both plant lines had similar pattern of root colonization under well-watered and drought stress conditions. In contrast, under drought stress, AM wildtype plants grew faster than mycorrhizal antisense plants. Plant gas exchange also appeared to depend on the expression of *NtAQP1* and paralleled the determined growth increments. The implications of enhanced symplastic water transport via NtAQP1 for the efficiency of the AM symbiosis under drought stress conditions are further discussed.

Key words: Aquaporin, antisense mutant, arbuscular mycorrhizae, drought, symbiotic efficiency.

5.1. Introduction

The arbuscular mycorrhiza is a symbiotic association between a plant root and a fungus. By this, the AM fungus occupies a protected ecological niche and receives plant photosynthates, while the plant improve their ability for nutrient uptake and tolerance to biotic and abiotic stresses (Smith and Read 1997). Among the abiotic stresses, water deficit is considered one of the most important factors limiting plant growth and yield (Kramer and Boyer 1997). Several eco-physiological studies investigating the role of AM symbiosis in protection against drought stress have demonstrated that the symbiosis often results in altered rates of water movement into, through and out of the host plants, with consequences on tissue hydration and plant physiology (for reviews see Augé 2001; Ruiz-Lozano 2003).

Some studies have demonstrated that AM symbiosis induces the expression of a gene encoding a tonoplast-located aquaporin in parsley and in alfalfa (Roussel *et al.* 1997; Krajinski *et al.* 2000). Aquaporins facilitate the membrane water transport along a water potential gradient. These proteins belong to the large major intrinsic protein (MIP) family of transmembrane proteins and are represented in all kingdoms (Chrispeels and Agre 1994). Two major classes of plant aquaporins, located in the plasma membrane or tonoplast,

respectively, have been identified so far (Johnson *et al*. 1990; Kammerloher *et al*. 1994). It has been suggested that vacuolar and plasma membrane aquaporins, acting in concert, are responsible for the cytosolic osmoregulation that is necessary for maintaining normal metabolic processes. However, inhibition studies of aquaporins *in vivo* and antisense mutant studies have also suggested that, in addition to cytosolic osmorregulation, aquaporins are important for the bulk flow of water in plants (Grote *et al*. 1998; Kjelbom *et al*. 1999; Martre *et al*. 2002 Javot *et al*. 2003). The high expression of genes encoding aquaporins in tissues involved in water transport suggests a role in transcellular water flow trough living cells (Barrieu *et al*. 1998; Aharon *et al*. 2003).

The NtAQP1 aquaporin of tobacco was isolated and characterized as a plasma membrane intrinsic aquaporin (Biela *et al*. 1999). Using RNA gel blot and whole mount hybridisation, Otto and Kaldenhoff (2000) found *NtAQP1* gene expression in almost all organs of tobacco, with the highest levels in the root. *In situ* immunological studies indicated NtAQP1 protein accumulation in the root exodermis and endodermis, in the cortex, close to the vascular bundles, in the xylem parenchyma, and in cells of the stomatal cavities. The aquaporin was found at sites of anticipated high water fluxes from and to the apoplast or symplast. In a subsequent report, Siefritz *et al*. (2002) could attribute the NtAQP1 function to cellular and whole-plant water relations.

Since AM fungi have been shown to enhance gene expression for tonoplast located aquaporins during root colonization, the goal of the present work was to investigate whether the impairment in *NtAQP1* gene expression affects the AM fungal colonization pattern and to find out if such an impairment has any effect on the symbiotic efficiency of AM fungi. The two objectives were investigated under well-watered and drought stress conditions in wildtype and antisense tobacco plants previously shown to have the *NtAQP1* gene expression reduced by 80% (Siefritz *et al*. 2002). Two AM fungi having diverse colonization patterns were examined, *Glomus mosseae* and *Glomus intraradices*.

5.2. Materials and Methods

5.2.1. Experimental design and statistical analysis

The experiment consisted of a three factors randomized complete block design of: (1) two tobacco plant lines, (2) mycorrhizal treatment consisting of two *Glomus* species or uninoculated control plants and (3) two water treatments (well-watered or drought stressed), with five replications per treatment totalling 60 pots (one plant per pot).

Within each water treatment, data were subjected to analysis of variance (ANOVA) with plant line, AM fungus and plant line-AM fungus interaction as sources of variation, and followed by Duncan´s multiple range test (Duncan 1955). As percentage values do not follow a normal distribution (they cannot be less than 0 nor more than 100), they were arcsin transformed for normalization before statistical analysis.

5.2.2. 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 during 1 h for 3 consecutive days). 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.

Seeds of *Nicotiana tabacum* (wildtype and antisense mutant) were surface sterilized with 70% ethanol for 2 min and with 2.5% sodium hypochloride for 10 min, then washed several times with sterile water to remove any trace of chemical that could interfere in seed germination. After sterilization, wildtype seeds were sown on plates containing 1/2 MS medium (Murashige and Skoog 1962) and antisense seeds on plates containing the same medium supplemented with kanamycin (100 μg/ml) for selection of antisense plants. After ten days of incubation at 25 °C, seedlings were transferred to pots containing 500 g of the sterilized soil/sand mixture.

Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* 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 aliquots of each inoculum, with similar infective characteristics (an average of 50 propagules per gram according to the most probable number test), were placed below tobacco seedlings. This amount of inoculum was selected in preliminary tests as optimum to produce a good infection level according to the total amount of soil in the pot. Non-mycorrhizal treatments received the same quantity of autoclaved inoculum together with a 2-ml aliquot of a filtrate (< 20 μm) of the AM inoculum in an attempt to provide a general microbial population free of AM propagules.

5.2.3. 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 350 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 (Roth *et al.* 1992; White *et al.* 1994). Volumetric soil water content is the ratio between the volume of water present and the total volume of the soil sample. It is a dimensionless parameter, expressed either as a percentage (% vol) or as a ratio (m^3m^{-3}). For half of the plants water was supplied daily to maintain constant soil water content close to field capacity (17% volumetric soil moisture). For the other half of the plants water was supplied daily to maintain constant soil water content close to 70% field capacity (10% volumetric soil moisture) and maintained under such conditions during the entire experiment. In order to control the level

of water stress, the pot 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 maintain soil water content at the desired 17% (well-watered plants) or 10% (droughted plants) of volumetric soil moisture.

5.2.4. Parameters measured

5.2.4.1. Biomass production

At harvest (8 wk after planting) the root system was separated from the shoot and the fresh weights were recorded. Shoot dry weight was determined after drying in a forced draught oven at 70 °C for two days.

5.2.4.2. Symbiotic development

The roots were carefully washed and stained by the normal non-vital trypan blue (TB) staining of all fungal tissues (Phillips and Hayman 1970). Mycorrhizal development was evaluated by the method of Trouvelot *et al.* (1986) (for more information visit, http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). The colonization frequency (F%) is a ratio between colonized root fragments and total number of root fragments observed. It gives an estimation of the root length colonized by the fungus. The colonization intensity (M%) is an estimation of the amount of root cortex occupied by fungal structures. Finally, the arbuscule abundance (A%) gives an estimation of the arbuscule richness in root system. Four replicates per treatment were used.

5.2.4.3. Solute accumulation

At harvest, free proline and total soluble sugars (TSS) were extracted from 1 g fw leaves as described by (Bligh and Dyer 1959). The methanolic phase was used for quantification of both substances. Proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates *et al.* (1973). TSS were analyzed by 0.1 ml of the alcoholic extract reacting with 3 ml freshly prepared anthrone (200 mg anthrone + 100 ml 72% (w:w) H_2SO_4) and placed in a boiling water bath for 10 min according to Irigoyen *et al.* (1992). After cooling, the absorbance at 620 nm was determined in a Shimadzu UV-1603 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The calibration curve was made using glucose in the range of 20 to 400 μg/ml.

5.2.4.5. Gas exchange

The CO_2 exchange rate (CER), transpiration rate, instantaneous water use efficiency (WUE) and stomatal conductance were measured on the third leaf from each plant. Atmospheric CO_2 was measured 5 m above ground level. Photosynthetic photon flux density (PPFD) was 1180 μmol m^{-2} s^{-1} in order to ensure that no limitation in photon irradiance occurred. Light was provided by a halogen lamp (General Electric 300 PAR 56/WFL). A model LCA-3 portable, integrated

infrared CO_2 analyzer (Analytical Development Co., Hoddesdon, UK) was used for these determinations. Measurements were made 2 h after the light was turned on. Precautions were taken according to Long and Hällgren (1987) to prevent diurnal, intra-plant and inter-plant variations in plant gas exchange.

5.3. Results

5.3.1. Shoot and root biomass production

Under well-watered conditions uninoculated wildtype and antisense plants did not show any significant difference in shoot or root growth (Figure 1).

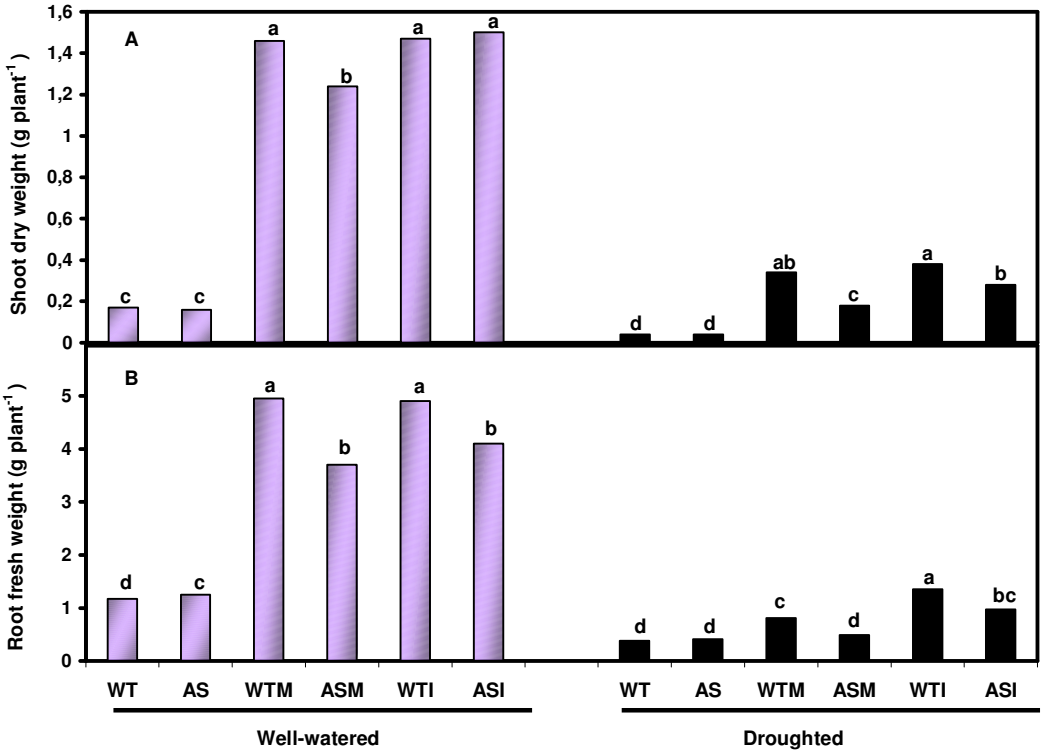

Figure 1. Shoot dry weight (A) and root fresh weight (B) in wildtype (WT) or antisense (AS) tobacco plants inoculated or not with *G. mosseae* (M) or *G. intraradices* (I) and cultivated under well-watered conditions or subjected to drought stress. Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple-range test ($n = 5$).

Inoculation with *G. mosseae* increased shoot biomass production more than 8-fold in wildtype plants and more than 7-fold in the antisense plants. Inoculation with *G. intraradices* also enhanced shoot biomass production in both wildype and antisense plants to a comparable level. The root fresh weigh was slightly more enhanced by mycorrhization in wildtype plants than in antisense plants (Figure 1).

Under drought stress conditions, uninoculated control plants of both phenotypes showed again a similar shoot and root biomass production and inoculation with both AM fungi increased shoot dry weight in both plant lines. In

any case, the increase in shoot dry weight was more effective in wildtype plants (8-fold) than the antisense plants (4-fold). In addition, for antisense plants under drought stress, *G. intraradices* enhanced more (55%) shoot biomass production than *G. mosseae*. Similarly, root fresh weight was not enhanced by *G. mosseae* in antisense plants.

5.3.2. AM colonization

After AM infection a similar colonization frequency (F) was obtained under all investigated treatments (Figure 2). In contrast to drought conditions, where similar values were observed, well-watered wild type plants inoculated with *G. mosseae* showed a slight decrease in F value. Colonization intensity (M) as well as arbuscule abundance (A) were lower in *G. mosseae*-colonized plants.

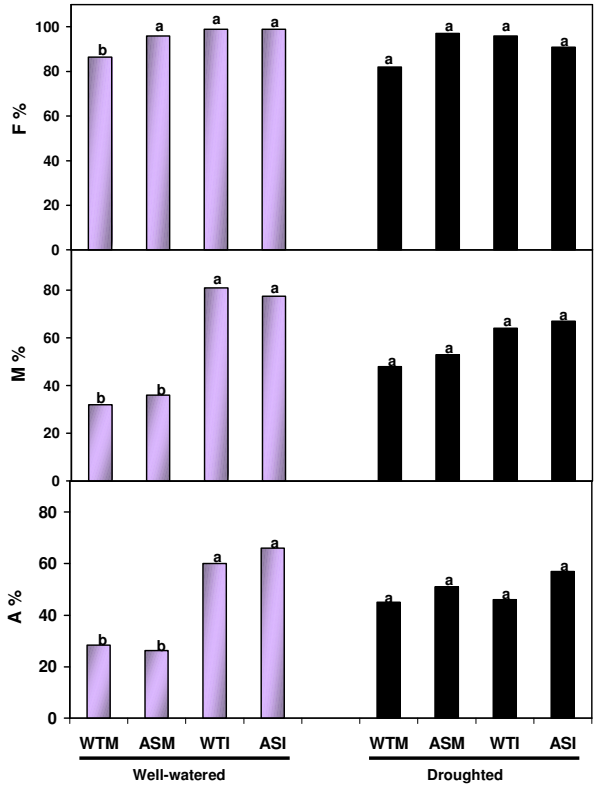

Figure 2. Frequency (F%) and intensity (M%) of root AM colonization, and arbuscule richness (A%) in wildtype (WT) or antisense (AS) tobacco plants inoculated or not with *G. mosseae* (M) or *G. intraradices* (I) and cultivated under well-watered conditions or subjected to drought stress. Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple-range test (n = 5).

5.3.3. Solute accumulation

Non-mycorrhizal wildtype and antisense plants accumulated more proline than mycorrhizal plants (Figure 3A). Under well-watered conditions, no differences in proline accumulation were observed among mycorrhizal treatments. In contrast, under drought stress antisense plants inoculated with either AM fungus accumulated more proline than the corresponding wildtype plants

inoculated with the same fungus. The increase was 200% for *G. mosseae*-colonized plants and 93% for *G. intraradices*-colonized plants.

TSS increased in both plant lines as a consequence of mycorrhization both under well-watered and under drought stress conditions (Figure 3B). The only exception was found in antisense plants colonized by *G. mosseae* that, under drought stress conditions showed equal soluble sugar accumulation than nonmycorrhizal plants.

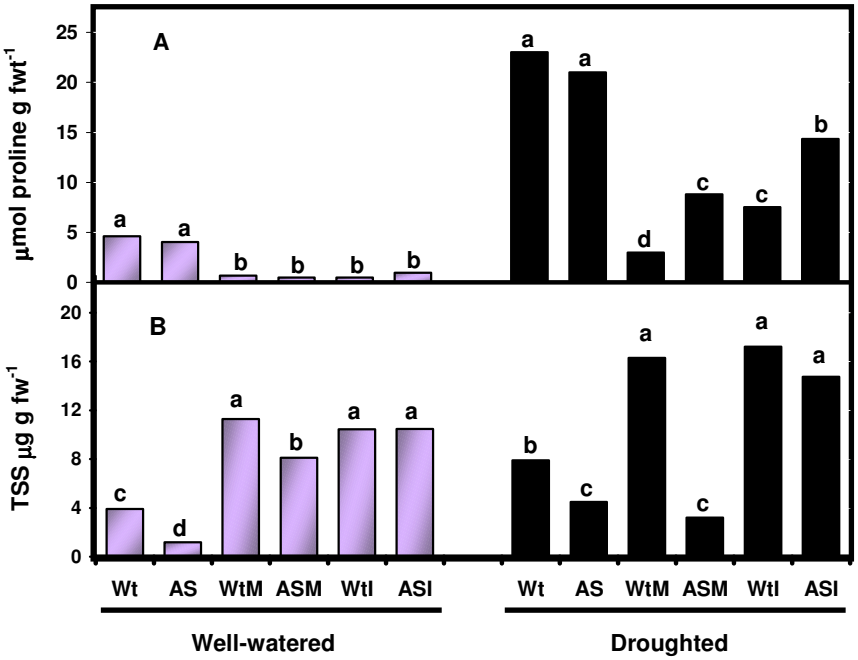

Figure 3. Proline (A) and total soluble sugar (B) accumulation in wildtype (WT) or antisense (AS) tobacco plants inoculated or not with *G. mosseae* (M) or *G. intraradices* (I) and cultivated under well-watered conditions or subjected to drought stress. Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple-range test ($n = 5$).

5.3.4. Gas exchange measurement

Mycorrhization enhanced leaf transpiration under all treatments (Table 1). Well-watered plant lines exhibited a similar transpiration rate after AM colonization. However, antisense lines colonized by *G. mosseae* displayed a slightly lower transpiration. Under drought, both mycorrhizal wildtype plants showed increased transpiration rates in comparison to the mycorrhizal antisense plants.

Like the transpiration rates, the CER was enhanced by mycorrhization (Table 1). Under well-watered conditions wildtype plants exhibited higher photosynthetic activity when colonized by *G. mosseae* than when colonized by *G. intraradices*. Antisense plants showed similar photosynthetic rate with both AM fungi. Under drought stress conditions, mycorrhizal wildtype plants had higher photosynthetic activity than the corresponding mycorrhizal antisense plants.

Under well-watered conditions, WUE was lower in wildtype plants colonized by *G. intraradices* than in nonmycorrhizal plants and no significant differences were observed among the rest of treatments (Table 1). In contrast, under drought stress, both plant lines colonized by *G. intraradices* showed the highest WUE. No significant differences between mycorrhizal and nonmycorrhizal plants were found for each *G. mosseae*-inoculated plant line.

Stomatal conductance was also enhanced by mycorrhization in both plant lines (Table 1). Under well-watered conditions, no differences were found in wildtype plants as a consequence of the colonizing fungus. In contrast, in antisense plants *G. mosseae* produced the lowest stimulation of stomatal conductance, while *G. intraradices* produced the highest one. Under drought stress conditions, colonization by *G. mosseae* enhanced more stomatal conductance in wildtype than in antisense plants, while *G. intraradices* behave similarly with both plant lines.

Treatment	CER	Transpiration	WUE	Conductance
Well-watered				
WT	8.3 d	1.6 c	5.3 a	082 d
AS	7.2 d	1.9 c	3.9 bc	095 d
WTM	61.0 a	14.1 a	4.4 abc	580 b
ASM	48.6 b	10.7 b	4.6 ab	446 c
WTI	38.6 c	14.4 a	2.7 d	540 b
ASI	48.1 b	13.7 ab	3.5 cd	690 a
Significance of sources of variation				
Plant line (P)	***	*	*	***
Mycorrhizal fungus (F)	**	*	**	**
P x F	***	**	*	**
Droughted				
WT	2.0 d	1.0 d	2.0 c	40 d
AS	1.5 d	0.9 d	1.8 cd	30 d
WTM	9.3 b	3.9 a	2.4 b	180 a
ASM	3.2 c	2.0 c	1.6 d	85 c
WTI	11.2 a	3.9 a	2.9 a	150 b
ASI	8.7 b	2.8 b	3.1 a	140 b
Significance of sources of variation				
Plant line (P)	***	***	*	*
Mycorrhizal fungus (F)	**	*	**	**
P x F	***	**	*	**

Table 1. CO_2-exchange rate (CER, µmol CO_2 m^{-2} s^{-1}), transpiration (mmol H_2O m^{-2} s^{-1}), water use efficiency (WUE, mmol CO_2/ mol H_2O) and stomatal conductance (mmol H_2O m^{-2} s^{-1}) in wildtype (WT) or antisense (AS) tobacco plants inoculated or not with *G. mosseae* (M) or *G. intraradices* (I) and cultivated under well-watered conditions or subjected to drought stress. Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple-range test ($n = 5$). Significance of the sources of variation is also displayed. * $P \leq 0.05$; ** $P \leq 0.01$; * **$P \leq 0.001$; ns, not significant.

5.4. Discussion

The AM system is an excellent example for the extensive morphological alterations that plant root cells undergo in order to accommodate the presence of symbionts. Cytoskeleton elements are rearranged, the nucleus increases in size,

amyloplasts lose their starch content and changes occur in the membrane systems of arbuscule-containing cells. The plant plasma membrane extends to form a novel periarbuscular membrane, which closely surrounds the fungal hyphae resulting in an estimated 3- to 10-fold increase in the outer plant cell surface (Bonfante and Perotto 1995; Gianinazzi-Pearson 1996).

Since most of the mycorrhiza-induced changes in plant root cells concern cytoplasmic or vacuolar membrane systems, a variation of expression patterns concerning genes that encode membrane-associated proteins can be expected (Krajinski *et al*. 2000). Conformingly, it has been shown that in mycorrhizal roots gene activity for plant-encoded aquaporins is upregulated and expression is localized in the highly compartmented vacuole of arbuscule-containing cells (Roussel *et al*. 1997; Krajinski *et al*. 2000). It has been proposed that these aquaporins probably optimize nutrient and water exchange between both symbiotic partners. They may also permit efficient osmoregulation of the highly compartmented root cells (Maurel *et al*. 2003). Considering that, and the fact that the periarbuscular membrane is derived from the plasmamembrane rather than the tonoplast, we have studied the effect of the plasma-membrane intrinsic aquaporin NtAQP1 on the pattern of mycorrhizal colonization and/or on the fungal symbiotic efficiency. Data from this study show that plant lines with a reduced or a natural level of NtAQP1 had similar pattern of root colonization under well-watered and drought stress conditions. The differences found under well-watered conditions concern the AM fungal species involved in the symbiosis. *G. mosseae* showed lower colonization intensity (M) and arbuscule abundance (A) than *G. intraradices* as has been observed before (Graham *et al*. 1996). With regard to colonization efficiency *G. intraradices* is a more aggressive AM fungus than *G. mosseae*. The lack of effect of the impairment in *NtAQP1* gene expression on the AM fungal colonization ability would suggest either that NtAQP1 function is irrelevant for the process of root colonization or it may suggest that the impairment in *NtAQP1* gene expression has been compensated by other mechanisms such as by changing the abundance or the activity of other aquaporins (Eckert *et al*. 1999; Johansson *et al*. 2000). However, the possibility that the reduced *NtAQP1* gene expression can be compensated by changing the abundance or the activity of other aquaporins has been ruled out. Siefritz *et al*. (2002) analized the consequences of antisense *NtAQP1* on other aquaporins expression and they found that only the closely related *NtPIP1a* gene showed reduced expression, although less severe than that of *NtAQP1* gene. The RNA levels of other aquaporin genes belonging to different subfamilies were unaffected by antisense *NtAQP1* expression. Thus they suggested that results obtained by subsequent analysis of the antisense plants using plant physiology techniques can only be ascribed to the function of *NtAQP1* and closely related *PIP1* genes.

Plant growth and development depend strongly on water absorption from the soil and its movement from the roots to other plant parts. Moreover, plant water status is important not only for growth under favourable environmental conditions; the ability of plants to tolerate water deficits and high salt levels also depends heavily on their water status, which is altered in response to

environmental conditions (Aharon *et al*. 2003). Long-distance water transport is carried out in the vascular tissues, where water is transported by bulk flow and membrane barriers are in most cases non-existent. In contrast, short-distance transport and transport in non-vascular tissues frequently involve transport across membranes, which include transport through proteinaceous water channels aquaporins. It has been proposed that rapid transmembrane water flow is possible due to the presence of aquaporins, and that the rate of water flux may be controlled by changing the abundance or the activity of the aquaporins (Eckert *et al*. 1999; Johansson *et al*. 2000).

In this study, both plant lines were cultivated under well-watered conditions and under drought stress. Under well-watered conditions uninoculated plants (antisense and wildtype) showed very similar growth rate or proline and TSS accumulation. Shoot biomass production improved drastically in both plant types after mycorrhizal colonization and the accumulation of TSS in mycorrhizal plants could have contributed to such an effect (Kameli and Lösel 1993). Under drought stress, however, mycorrhizal wildtype plants grew faster than mycorrhizal NtAQP1 antisense plants, indicating that the symbiotic efficiency of both AM fungi (in terms of plant biomass production) was greater with wildtype than with antisense plants. The lower symbiotic efficiency of AM fungi with AS plants was evidenced not only under drought stress, but also under well-watered conditions in the case of *G. mosseae*. Mainly when the colonizing fungus was the more aggressive *G. intraradices* the ability of the plant to increase membrane water permeability by NtAQP1 seems to be beneficial in terms of biomass production. The ability of the plant for a rapid growth as an adaptation to changed environmental conditions could indeed be assured by the function of NtAQP1. Other examples were provided by Siefritz *et al*. (2004) in studies about developmental regulation and function in growth processes of NtAQP1. The plant gas exchange also appears to depend on the expression of *NtAQP1*, because increase and decrease of transpiration, photosynthetic activity and stomatal conductance parallels the determined growth increments. In recent works, Aharon *et al*. (2003) and Uehlein *et al*. (2003) showed that heterologous respectively homologous over-expression of an *Arabidopsis* plasma membrane aquaporin or *NtAQP1* in tobacco significantly increased plant growth rate, transpiration rate, stomatal density and photosynthetic efficiency when plants were cultivated under favourable growth conditions. In contrast to our results, such over expression had no beneficial effect under salt stress and was deleterious during drought (Aharon *et al*. 2003). However, the status of mycorrhization was not determined in these experiments and artificial increase of membrane water permeability by over expression of an arbitrarily chosen aquaporin gene in all tissues of the plant could have unpredictable effects on plant water relations and also on cell stability.

The lower proline content in wildtype plants can be an indication that these plants had a better water status and needed lower osmotic adjustment. In contrast, antisense plants had to accumulate more proline in order to decrease osmotic potential in their tissues (more active osmorregulation, suggesting an also possibly lower water status). This agree with the fact that nonmycorrhizal plants

always accumulated more proline (mainly under drought stress), indicating that they were more strained by drought stress than mycorrhizal plants as has been reported previously (Augé 2001; Ruiz-Lozano 2003).

Taken together, the present results indicate that enhanced symplastic water transport via the plasma membrane aquaporin NtAQP1 is important for the efficiency of AM symbiosis, at least under drought stress conditions. This confirms the results obtained by Siefritz *et al.* (2002), which show a measurable and visible effect of *NtAQP1* expression under drought stress and the report by Ruiz-Lozano (2003) indicating that AM symbiosis contributes to the modulation of the total amount of water, thereby modifying plant water status and plant growth.

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5.6. References

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EXPRESIÓN DE GENES PIP DE AQUAPORINAS EN PLANTAS MICORRÍCICO ARBUSCULARES DE *Glycine max* Y *Lactuca sativa* EN RELACIÓN CON LA TOLERANCIA AL ESTRÉS HÍDRICO

Resumen

Si bien es cierto que el descubrimiento de las aquaporinas en plantas ha significado un cambio paradigmático en el conocimiento de las relaciones hídricas en las plantas, la relación existente entre aquaporinas y las respuestas de las plantas a la sequía aún no está clara. Además, hasta ahora no se ha investigado la contribución de los genes de aquaporinas a la mejora de la tolerancia a la sequía en plantas MA. Por lo tanto hemos estudiado a nivel molecular si la expresión de los genes que codifican aquaporinas en raíces está alterada por la simbiosis MA como mecanismo para mejorar la tolerancia al déficit hídrico en la planta hospedadora. En este estudio se clonaron varios genes que codifican aquaporinas de membrana plasmática (PIPs) de soja y lechuga y se estudió su patrón de expresión en plantas MA y control cultivadas bajo condiciones óptimas de riego o en condiciones de déficit hídrico. Los resultados mostraron que las plantas MA respondieron al estrés hídrico inhibiendo la expresión de los genes PIP estudiados así como anticipando su inhibición en relación a las plantas controles. Las posibles implicaciones fisiológicas de la inhibición de estos genes PIP como mecanismo para disminuir la permeabilidad de la membrana y permitir la conservación de agua en las células son tratados más en detalle.

Palabras clave: simbiosis micorrícico arbuscular, tolerancia a la sequía, aquaporina PIP, déficit hídrico.

PIP AQUAPORIN GENE EXPRESSION IN ARBUSCULAR MYCORRHIZAL *Glycine max* AND *Lactuca sativa* PLANTS IN RELATION TO DROUGHT STRESS TOLERANCE

Abstract

Although the discovery of aquaporins in plants has resulted in a paradigm shift in the understanding of plant water relations, the relationship between aquaporins and plant responses to drought still remains elusive. Moreover, the contribution of aquaporin genes to the enhanced tolerance to drought in AM plants has never been investigated. Therefore, we studied, at a molecular level, whether the expression of aquaporin-encoding genes in roots is altered by the AM symbiosis as a mechanism to enhance host plant tolerance to water deficit. In this study, genes encoding plasma membrane aquaporins (PIPs) from soybean and lettuce were cloned and their expression pattern studied in AM and non-AM plants cultivated under well-watered or drought stressed conditions. Results showed that AM plants responded to drought stress by down-regulating the expression of the *PIP* genes studied and anticipating its down-regulation as compared to non-AM plants. The possible physiological implications of this down-regulation of *PIP* genes as a mechanism to decrease membrane water permeability and to allow cellular water conservation is further discussed

Key words: *arbuscular mycorrhizal symbiosis, drought tolerance, PIP aquaporin, water deficit*

6.1. Introduction

The arbuscular mycorrhizal (AM) symbiosis is the visible result of the interaction between a plant root and a fungus. By this, the AM fungus occupies a protected ecological niche and receives plant photosynthates, while plants improve their ability for nutrients uptake and their tolerance to biotic and abiotic stresses (Smith and Read, 1997). Among the abiotic stresses, 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 (Kramer and Boyer, 1997; Sheng *et al.*, 2004). Several eco-physiological studies investigating the role of AM symbiosis in protection against drought stress have demonstrated that the symbiosis often results in altered rates of water movement into, through and out of the host plants, with consequences on tissue hydration and plant physiology (for reviews see Augé, 2001, 2004; Ruiz-Lozano, 2003).

The AM system is an excellent example for the extensive morphological alterations that plant root cells undergo in order to accommodate the presence of

symbionts. Cytoskeleton elements are rearranged, the nucleus increases in size, amyloplasts lose their starch content and changes occur in the membrane systems of arbuscule-containing cells. The plant plasma membrane extends to form a novel periarbuscular membrane, which closely surrounds the fungal hyphae resulting in an estimated 3- to 10-fold increase in the outer plant cell surface (Bonfante and Perotto 1995; Gianinazzi-Pearson, 1996). Since most of the mycorrhiza-induced changes in plant root cells concern cytoplasmic or vacuolar membrane systems, a variation of expression patterns concerning genes that encode membrane-associated proteins can be expected (Krajinski *et al.,* 2000). Accordingly, it has been shown that in mycorrhizal roots a gene encoding for a plant aquaporin is up-regulated and the expression is localized in the highly compartmented vacuole of arbuscule-containing cells (Roussel *et al.,* 1997; Krajinski *et al.,* 2000). Several aquaporin-encoding genes have also been shown to be up-regulated in ectomycorrhizal poplar plants, and this was correlated with an increased water transport capacity of mycorrhizal poplar roots (Marjanovic *et al.,* 2005). Finally, it has been also 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.,* 2005a).

Aquaporins are water channel proteins that facilitate and regulate the passive movement of water molecules down a water potential gradient. These proteins belong to the large major intrinsic protein (MIP) family of transmembrane proteins and are represented in all kingdoms (Chrispeels and Agre 1994; Maurel, 1997; Tyerman *et al.,* 2002). Two major classes of plant aquaporins, located in the plasma membrane (PIPs) or tonoplast (TIPs), respectively, have been identified so far (Johnson *et al.,* 1990; Kammerloher *et al.,* 1994). Other two classes of plant aquaporins are the homologues to the soybean Nodulin-26 aquaporin (NIPs) and the small basic intrinsic proteins (SIPs). The localization and function of SIPs are unknown at the moment (Johanson *et al.,* 2001; Luu and Maurel, 2005).

The rate of water flux into or out of a cell is determined by the water potential gradient that acts as the driving force for transport and by the water permeability of the membrane. Aquaporin proteins facilitate osmosis by forming water-specific pores as an alternative to water diffusion through the lipid bilayer, thus increasing the water permeability of the membrane (Schäfner, 1998; Kjelbom *et al.,* 1999; Smart *et al.,* 2001). It has been suggested that vacuolar and plasma membrane aquaporins, acting in concert, are responsible for the cytosolic osmoregulation that is necessary for maintaining normal metabolic processes (Kjelbom, 1999). Moreover, inhibition studies of aquaporins *in vivo* and antisense transgenic studies have also suggested that aquaporins are crucial for the bulk flow of water in plants (Grote *et al.,* 1998; Kjelbom *et al.,* 1999; Martre *et al.,* 2002; Siefritz *et al.,* 2002; Javot *et al.,* 2003).

The discovery of aquaporins in plants has caused a significant change in the understanding of plant water relations. However, the relationship that exists between aquaporins and plant responses to drought still remains elusive and with contradictory results (Aharon *et al.,* 2003; Lian *et al.,* 2004). Moreover, the

contribution of aquaporin genes to the enhanced tolerance to drought in AM plants has never been investigated. Krajinski *et al.* (2000) proposed that the up-regulation of aquaporins by the AM symbiosis probably optimizes nutrient and water exchange between both symbiotic partners. They may also permit efficient osmoregulation of the highly compartmented root cells (Maurel *et al.,* 2002). However, the studies by Roussel *et al.* (1997) and Krajinski *et al.* (2000) were carried out under well-watered conditions and they did not test the expression of the aquaporin gene in AM plants under drought stress conditions.

Many studies have suggested that aquaporins contribute significantly to the hydraulic conductivity of cells and that they have a role in cellular osmoregulation (Kjelbom *et al.,* 1999; Martre *et al.,* 2002; Javot *et al.,* 2003). In recent years, much effort has been concentrated on investigating the function and regulation of PIP aquaporins. These aquaporins seem to play a specifically important role in controlling transcellular water transport. For instance, they are abundantly expressed in roots where they mediate most of soil water uptake (Javot and Maurel, 2002) and transgenic plants down-regulting one or more PIP genes had lower root water uptake capacity (Siefritz *et al.,* 2002; Javot *et al.,* 2003). Since aquaporins are regulated both at transcriptional and activity levels (Martre *et al.*, 2002), we have considered of interest to study whether the expression of aquaporin-encoding genes in roots is altered by the AM symbiosis as a mechanisms to enhance host plant tolerance to water deficit. To achieve this, genes encoding plasma membrane aquaporins (PIPs) from soybean and lettuce were cloned and their expression pattern studied, in AM and non-AM plants cultivated under well-watered or drought stress conditions by using northern blot and quantitative real-time PCR. At the same time, PIP protein abundance was studied by western blot.

6.2. Materials and Methods

6.2.1. Experimental designs

First experiment with Glycine max

The experiment consisted of a randomized complete block design with three inoculation treatments: (1) non-inoculated control plants (NI), (2) plants inoculated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, strain USDA 110 (Br) and (3) plants inoculated with the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *B. japonicum* (Gm+Br). The use of these three inoculation treatments was decided because legume plants, in their natural state, are nodulated. Hence, the non-AM control plants were those inoculated only with *B. japonicum* (Br treatment). However, we also included an absolute non-inoculated control treatment for comparison. Twelve replicates of each microbial treatment were done, totalling 36 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 days before harvest (35 days after inoculation).

Second experiment with Glycine max

The experiment consisted of a randomized complete block design with the same inoculation treatments described for the first experiment: For each treatment plants were harvested at four time intervals: 5, 12, 20 or 35 days after inoculation (dai). There were different number of replicates for each treatment, ranging from 12 replicates for plants harvested after only 5 days, to 6 replicates for plant harvested after 35 days, totalling 108 pots (one plant per pot). Half of the plants were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 5 days (for plants harvested 5 dai) or for 10 days (for the rest of treatments) before harvest.

Experiment with Lactuca sativa

The experiment consisted of a randomized complete block design with three inoculation treatments: (1) non-inoculated control plants (NI), (2) plants inoculated with the AM fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (Gm) and (3) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) (Gi). There were ten replicates of each treatment, totalling 30 pots (one plant per pot), so that half of them were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 10 days 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.

6.2.2. 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 days). 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_2O_2 solution for 8 min, then washed several times with sterile water to remove any trace of chemical that could interfere in 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 *Zea mays* 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 80 infective propagules per gram, according to the most probable number 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 together with a 2-ml aliquot of a filtrate (< 20 μm) of the AM inoculum in order to provide a general microbial population free of AM propagules.

6.2.3. Choice of plant and fungal species

The choice of *Glycine max* and *Lactuca sativa* is based on the fact that both species are highly mycotrophic and responsive to drought stress, thus representing a good system to study the effects of the AM symbiosis when coping with drought stress.

The choice of *G. mosseae* and *G. intraradices* as mycorrhizal fungi is based on the fact that they have a clearly different physiological behaviour, including symbiotic efficiency and colonization pattern of the host root. In addition, these fungi have a clearly different ability to improve plant water uptake under drought stress, as has been evidenced though a number of studies (Ruiz-Lozano *et al.,* 1995; Tobar *et al.,* 1994a,b; Marulanda *et al.,* 2003).

6.2.4. 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) as previously described (Porcel and Ruiz-Lozano, 2004; Porcel *et al.,* 2004, 2005b). Four weeks after planting half of the plants were allowed to dry until soil water content reached 70% field capacity, while the other half were maintained at field capacity. Plants were maintained under such conditions for 10 days. 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 keep the soil water content at the desired level (Porcel and Ruiz-Lozano, 2004; Porcel *et al.,* 2004, 2005b). For the second experiment, half of the plants were maintained at field capacity during the entire experiment, while the other half were drought stressed as indicated above for 5 days (plants harvested 5 dai) or for 10 days for the rest of 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_2HPO_4 and 2 mM NH_4NO_3 (NI plants), 0.35 mM K_2HPO_4 (Br 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 fertilization level for non-mycorrhizal plants was meant to obtain well-watered control plants of similar size and nutrient contents to the AM plants tested in this assay.

6.2.5. 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 mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980). Nodule number in soybean roots was determined using a dissecting microscope.

6.2.6. Relative water content

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

6.2.7. Leaf water potential

The leaf water potential (Ψ) was determined one day before harvest with a C-52 thermocouple psychrometer chamber and a HR-33T dew point microvoltmeter (Wescor Inc, Logan, UT, USA). Leaf discs were cut, placed inside the psychrometer chamber and allowed to reach temperature and water vapour equilibrium for 30 min before measurements were made by the dew point method.

6.2.8. 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.

6.2.9. Cloning the *GmPIP* and *LsPIP* genes

Several stretches of conserved amino acids were apparent from the compilations of sequences for aquaporins in plants and fungi. Two stretches were used to design degenerate oligonucleotide primers as described by Numberg *et al.* (1989): primer forward 5' CA(CT) AT(CA) AAC CC(AG) GC(GA) GTG AC-3' and primer reverse 5'-C CAT GAA (AC)AC (AC)GC AAA (TA)CC (AG) AT-3'. Using cDNA from either soybean or from lettuce roots subjected to drought stress as template, a cDNA fragment of about 335 bp was amplified with these primers and the polymerase chain reaction (PCR). PCR was carried out as described previously (Porcel *et al.,* 2004, 2005b). 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 pGEM 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).

6.2.10. 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 Centre for Biotechnology Information (NCBI).

6.2.11. Northern blot analysis

Northern blot with *GmPIP1*, *GmPIP2*, *LsPIP1* or *LsPIP2* probes were carried out as previously described (Ruiz-Lozano *et al.*, 2002; Porcel *et al.*, 2004, 2005b). Hybridizations were carried out overnight at 65 °C under standard conditions (Sambrook *et al.*, 1989). After washing twice for 5 min at room temperature in 2X SSC and 0.1% SDS, and twice for 15 min at 65 °C with 0.5X SSC and 0.1% SDS, membranes were exposed overnight to Kodak X-RAY-OMAT at –70 °C. Before performing the northern blot, equal RNA loading and transfer to the nylon membranes were verified by methylene blue staining of the membranes (Herrin and Schmidt, 1988). The amount of rRNA in these membranes was quantified

using Quantity One software (BioRad, Hemel Hempstead, UK). After the northern blot the signals on autoradiograms were analyzed and quantified using the same software. Transcript accumulation levels for each gene probe (in arbitrary units) were divided by the corresponding amount of rRNA in the membrane (also in arbitrary units). Each quantification of signals on autoradiograms and of rRNA in the membranes was repeated three times and the average value for each was used for normalization. Northern blot analyses were repeated two times with different set of plants.

6.2.12. Quantitative real-time RT-PCR

GmPIP2 and LsPIP2 gene expression was studied by real-time PCR by using iCycler (Bio-Rad, Hercules, California, USA). 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, 1x first strand buffer (Invitrogen, Carlsbad, California, USA) and 200 U of Superscript II Reverse Transcriptase (Invitrogen). The primer sets used to amplify *GmPIP2 and LsPIP2 genes* in the synthesized cDNAs are shown in Table 1. 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 1x SyBR Green (Molecular Probes, Eugene, Oregon, USA), and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) in 1x PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl).

Primer	5' 3'
AQPGmFor	TTGGCGAGGAAGTTGTCGTTGC
AQPGmRev	AGATCCAGTGTTCATCCCAACC
18SGmFor	CGATCAGATACCGTCCTAGTC
18SGmRev	CCAACTAAGAACGGCCATGCACC
AQPLsFor	CAAATGGTCCTTCTACAGAGC
AQPLsRev	CAAACACTGTGCAATCATGTATCC
18SLsFor	CAGGTCCAGACATAGTAAGA
18SLsRev	GACCATTCAATCGGTAGGAGC

The annealing temperature used for all primer sets from soybean was 60 ºC and 56 ºC for primers used with lettuce.

Table 1. Primers used in this study.

The PCR program consisted in 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 procedure was checked with a heat dissociation protocol (from 70 °C 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 18S shown in Table 1.
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 (Livak and Schmittgen, 2001). Negative controls without cDNA were used in all PCR reactions.

6.2.13. Western blot analysis

The microsome purification, SDS-PAGE gel, transferring proteins to nitrocellulose membrane, and blocking were all carried out as described by Aroca *et al.* (2005). The membranes were incubated in Tris-buffered-saline buffer (TBS) with 0.05% Tween 20 in presence of PIP1 or PIP2 antibodies from *Arabidopsis thaliana* (Kammerloher *et al.,* 1994; Daniels *et al.,* 1994). Each antibody was incubated 1:500 dilution overnight at 4 °C and the secondary antibody (Mouse anti-chicken IgG coupled to horseradish peroxidase; Sigma) 1:5,000 1 h at room temperature. The signal was developed using a chemiluminescent substrate (West-Pico, Super Signal; Pierce, Rockford, IL). Quantification of inmunoblots was carried out as described previously (Aroca *et al.*, 2005).

6.2.14. Nucleotide sequence accession number

The nucleotide sequences corresponding to *GmPIP1, GmPIP2, LsPIP1* and *LsPIP2* cDNAs have been deposited in the EMBL database under accession numbers AJ937960, AJ937961, AJ937962 and AJ937963, respectively.

6.3. Results

6.3.1. Symbiotic development in soybean and lettuce plants

No AM colonization or nodules were observed in non-inoculated soybean plants. In the first experiment with soybean the percentage of AM infection was near 65% (Br+Gm plants), with no significant differences between well-watered and drought stressed conditions. The number of nodules in *B. japonicum*-inoculated plants ranged from 30 to 50 (data not shown). In the second experiment with soybean (time-course experiment) mycorrhizal infection inside roots and nodule formation were visible 12 dai and both symbioses were progressing until the last harvest (35 dai). The AM colonization at 12 dai was 17% (ww) and 15% (ds) of mycorrhizal root length, at 20 dai it reached 30% (ww) and 25% (ds) and at 35 dai it was 55% (ww) and 47% (ds), while the number of nodules ranged from 20 to 30 in *B. japonicum*-inoculated plants (data not shown).

In lettuce (third experiment), the percentage of AM colonization was near 70% for *G. mosseae*-inoculated plants and near 84% for *G. intraradices*-inoculated plants. The AM infection resulted unaffected by drought stress (data not shown).

6.3.2. Relative water content

Soybean plants from the first experiment showed no significant differences in RWC when cultivated under well-watered conditions (Figure 1B). Drought stress decreased the RWC in all the treatments, but AM plants maintained a significantly higher RWC than both non-AM treatments.

Soybean plants from the time-course experiment also showed no significant differences in their relative water content when cultivated under well-watered conditions (Figure 2B). Drought decreased their RWC in all treatments but at 20 and 35 dai AM plants showed higher RWC than non-AM plants. Lettuce plants showed a similar trend, with no significant differences in RWC under well-watered conditions and higher RWC of AM plants than non-AM plants when subjected to drought (Figure 3B).

6.3.3. Leaf water potential (Ψ)

The leaf water potential of soybean (first experiment) and lettuce plants (third experiment) was unaffected by the microbial treatment when cultivated under well-watered conditions (Table 2). In contrast, under drought stress conditions Ψ was higher (less negative) in AM plants than in non-AM plants.

	Ψ	
Treatment	Well-watered	Droughted
Soybean		
NI	-1.4c	-2.4a
Br	-1.6c	-2.3a
Gm+Br	-1.5c	-1.9b
Lettuce		
NI	-1.1c	-2.0a
Gm	-1.1c	-1.6b
Gi	-1.3c	-1.7b

Tabla 2. Leaf water potential (Ψ, MPa) in soybean and lettuce plants. Treatments for soybean are designed as NI, noninoculated controls; Br, *Bradyrhizobium japonicum*; Br+Gm, *B. japonicum* plus *G. mosseae* and for lettuce as NI, noninoculated controls; Gm, *Glomus mosseae* and Gi, *Glomus intraradices*. Plants were either well-watered or drought stressed for 10 days.

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

6.3.4. Cloning *GmPIP* and *LsPIP* genes

The use of the degenerate primers for aquaporins allowed us to obtain several clones, which contained inserts of the expected size using cDNA from soybean and from lettuce roots. The sequencing of four of the clones obtained from soybean cDNA showed that all of them contained a cDNA insert putatively encoding for aquaporins. These four clones corresponded to two different sequences named *GmPIP1* and *GmPIP2*. The first clone (*GmPIP1*) contained a cDNA fragment of 335 bp encoding for a putative protein of 88% identity with PIP1 from *Medicago truncatula* (accession Q946J9, e = $1e^{-109}$). The second clone (*GmPIP2*) contained a cDNA fragment of 331 bp encoding for a putative protein of 83% identity with a PIP2 from *Zea mays* (accession Q9ATM4, e = $7e^{-46}$). The homology between *GmPIP1 and GmPIP2* nucleotide sequences was 68%.

In the case of lettuce, another four clones were sequenced, that corresponded to two different sequences putatively encoding for aquaporins. Such clones were named *LsPIP1* and *LsPIP2*. The first clone (*LsPIP1*) contained a cDNA fragment of 334 bp. The putative protein encoded by this cDNA gave 92% identity with a PIP1 from *Vitis berlandieri* (accession Q9M7B2, e = $4e^{-52}$). The second clone (*LsPIP2*) contained a cDNA fragment of 331 bp. The putative protein encoded by this cDNA gave 93% identity with a PIP2 from *Vitis vinifera* (accession Q5PXH0, e = $2e^{-54}$). The homology between *LsPIP1 and LsPIP2* nucleotide sequences was 75%.

6.3.5. Northern blot analysis with soybean RNAs

Both cDNA inserts from soybean (*GmPIP1* and *GmPIP2*) were used as probes in northern blot analyses with soybean roots RNA from non-mycorrhizal and mycorrhizal treatments (see experimental design). The expression of both genes resulted down-regulated by drought stress (Figure 1).

GmPIP1 showed the highest expression level in plants cultivated under well-watered conditions. The gene expression corresponding to non-inoculated control plants was set as 100% in arbitrary units after normalization according to the amount of ribosomal RNA loaded in the blots. The three treatments decreased in a similar way (by 25-30%) *GmPIP1* gene expression when cultivated under drought stress conditions. A similar trend was found for *GmPIP2* gene expression (Figure 1), with the exception of nodulated control plants (Br) that exhibited a decreased gene expression also under well-watered conditions. Drought stress decreased *GmPIP2* transcript accumulation in a higher extent than for *GmPIP1*, ranging from 45% decrease in NI plants to 60% decrease in AM plants.

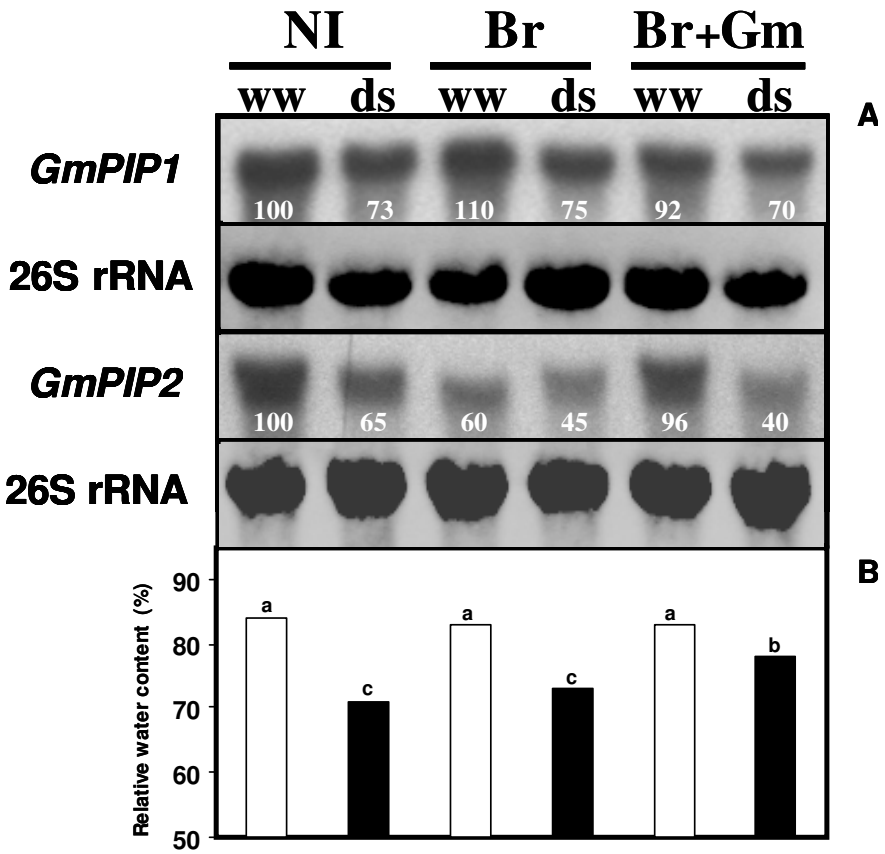

Figure 1. A. Northern blot of total RNA (15 µg) from soybean roots using *GmPIP1* and *GmPIP2* gene probes. Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Br+Gm, *B. japonicum* plus *G. mosseae*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The lower panels show the amount of 26S rRNA loaded for each treatment (methylene blue staining). Numbers close to each northern represent the relative gene expression (after normalization to rRNA) as a percentage of the value for well-watered noninoculated plants. **B**. Relative water content of soybean plants grown under well-watered conditions (white column) or subjected to drought stress (black column).

6.3.6. Quantitative real-time RT-PCR in soybean

As both genes showed a similar pattern of gene expression in AM versus non-AM plants and also in drought stressed versus well-watered plants, we designed specific primers only for one of the genes in order to follow its expression pattern also by quantitative real-time PCR. We selected *GmPIP2* since there are evidences that PIP2 are more active in water flow across plasma membranes than PIP1 (Chaumont *et al.,* 2000; Fetter *et al.,* 2004; Bots *et al.,* 2005). The data on gene expression obtained for plants from the first experiment (Figure 4) corroborated the pattern of gene expression found with northern blot. In fact, non-inoculated plants showed the highest gene expression under well-watered conditions and an important decrease under drought stress. Nodulated non-AM plants (Br) showed again a reduced gene expression level even under well-watered conditions, while AM plants also showed higher *GmPIP2* gene expression under well-watered conditions and significant down-regulation under drought stress conditions.

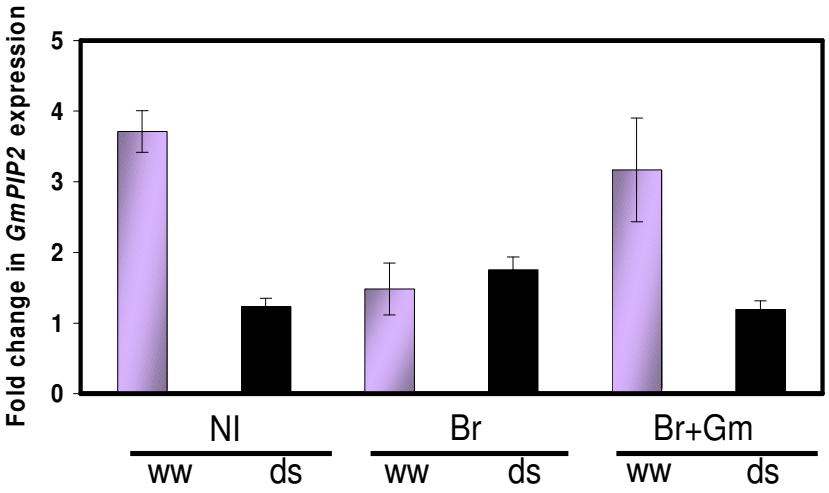

Figure 4. Fold change in *GmPIP2* gene expression determined by quantitative real-time PCR using gene-specific primers for *GmPIP2* and 18S rRNA. The fold change in *GmPIP2* gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Br+Gm, *B. japonicum* plus *G. mosseae*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. Data represent the mean of five replicates. Bars represent SE.

6.3.7. Time-course analysis of *GmPIP2*

Based on the results obtained by northern blot and by quantitative PCR which showed a down-regulation of PIP genes under drought stress conditions, but no important differences between non-AM and AM plants, we planned a time-course experiment in order to study the expression level of *GmPIP2* gene at different time intervals of the symbioses in soybean roots. Plants harvested at 5 dai showed little variation in *GmPIP2* transcript accumulation under any condition (Figure 2A).

Only the AM plants exhibited a higher down-regulation (by 20%) of *GmPIP2* gene expression under drought stress conditions than the rest of treatments. At 12 dai, the two non-AM treatments continued showing little variation in gene expression under all conditions. AM plants showed again a significant down-regulation (by 55%) of *GmPIP2* gene expression under drought stress conditions than the rest of treatments. At 20 dai, all the treatments showed down-regulation of *GmPIP2* gene under drought, but this down-regulation was lower for the two nonAM plants (the decrease ranged from 12% for NI plants to 40% for Br plants) than for the AM plants (the decrease was 71%). Finally, at 35 dai the pattern of *GmPIP2* gene expression was similar to that obtained in the first northern blot (Figure 1A) or by means of quantitative PCR (Figure 4). In fact, in all these cases plants were of the same age and at the same developmental stage. The most remarkable result was that under drought stress conditions non-inoculated plants down-regulated *GmPIP2* gene expression to a similar extent as AM plants. Nodulated control plants (Br) showed reduced gene expression even under well-watered conditions.

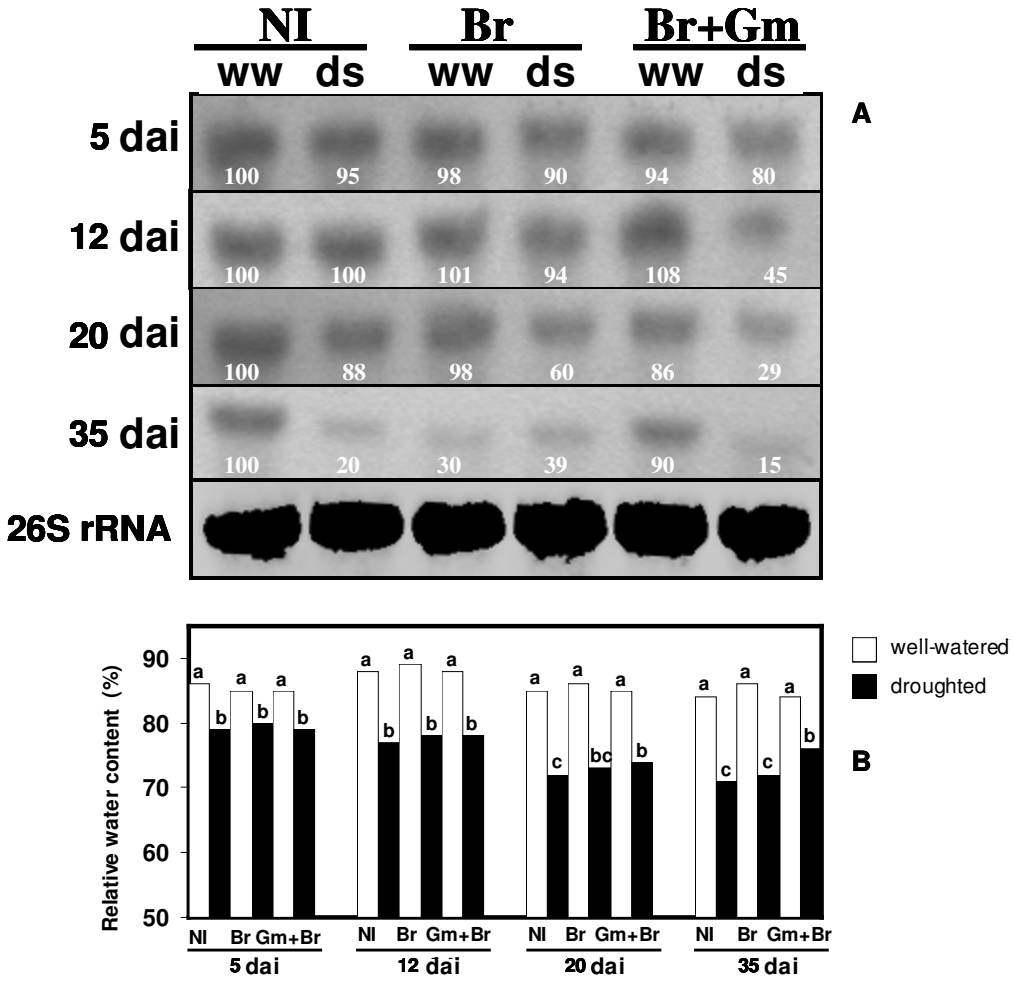

Figure 2. A. Northern blot of total RNA (15 µg) from soybean roots using *GmPIP2* gene probe. Plants were harvested 5, 12, 20 or 35 days after inoculation (dai). Treatments are designed as NI, noninoculated controls; Br, *Bradyrhizobium japonicum*; Br+Gm, *B. japonicum* plus *G. mosseae*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The lower panel shows a representative example of the amount of 26S rRNA loaded for each treatment (methylene blue staining). Numbers close to each northern represent the relative gene expression (after normalization to rRNA) as a percentage of the value for well-watered noninoculated plants. **B.** Relative water content of soybean plants grown under well-watered conditions (white column) or subjected to drought stress (black column).

6.3.8. Northern blot analysis with lettuce RNA

In order to test the behaviour of AQP genes in a non-legume plant and to avoid the interference of the AM symbiosis with the *Bradyrhizobium* symbiosis, the cDNAs cloned from lettuce (*LsPIP1* and *LsPIP2*) were used for northern blot analysis with RNA from non-inoculated or AM lettuce roots cultivated either under well-watered or drought stressed conditions.

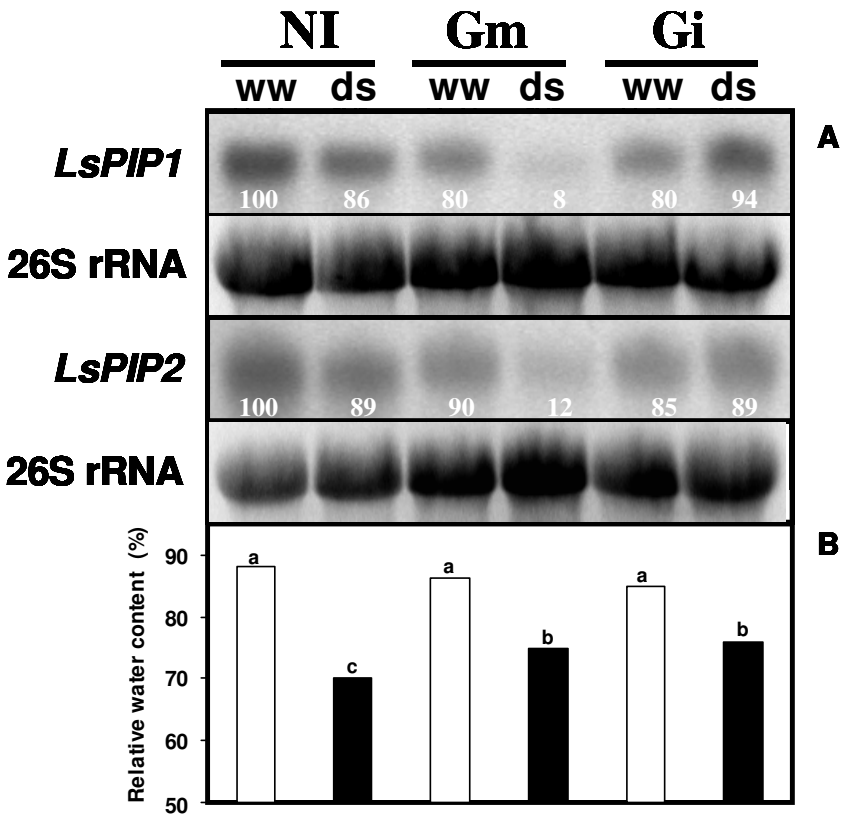

Figure 3. A. Northern blot of total RNA (15 µg) from lettuce roots, using *LsPIP1* and *LsPIP2* gene probes. Treatments are designed as NI, noninoculated controls; Gm, *Glomus mosseae* and Gi, *Glomus intraradices*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The lower panels show the amount of 26S rRNA loaded for each treatment (methylene blue staining). Numbers close to each northern represent the relative gene expression (after normalization to rRNA) as a percentage of the value for well-watered noninoculated plants. **B.** Relative water content of soybean plants grown under well-watered conditions (white column) or subjected to drought stress (black column).

Two AM fungi (*G. mosseae* and *G. intraradices*) with clearly different ability to improve plant water uptake under drought stress (Marulanda *et al.*, 2003) were used. Results showed that both lettuce *PIP* genes behave similarly (Figure 3A).

The highest gene expression was found in non-inoculated plants under well-watered conditions that was set as 100% in arbitrary units. Gene expression in these non-inoculated plants was slightly decreased by drought stress (14% decrease for LsPIP1 and 11% decrease for LsPIP2). Both AM treatments showed a reduced transcript accumulation for both PIP genes under well-watered conditions, as compared to non-inoculated plants. While G. mosseae-inoculated plants decreased drastically (by near 90%) the level of gene expression under drought stress conditions, G. intraradices-inoculated plants did not show such a decrease.

6.3.9. Quantitative real-time RT-PCR in lettuce

We designed specific primers only for one of the PIP genes in order to follow its expression pattern also by quantitative real-time PCR. As in the case of soybean, we selected *LsPIP2*.

Data from quantitative PCR experiments showed a similar pattern of *LsPIP2* gene expression to northern blot analysis, with the highest transcript accumulation in non-inoculated plants under well-watered conditions and down-regulation in these plants under drought stress conditions (Figure 5). Both AM treatments showed a lower transcript accumulation than non-AM plants and, again, *G. mosseae*-colonized plants showed a drastic down-regulation of *LsPIP2* gene expression under drought stress conditions. *G. intraradices*-colonized plants did not show such a down-regulation of gene expression under drought stress as compared to well-watered conditions.

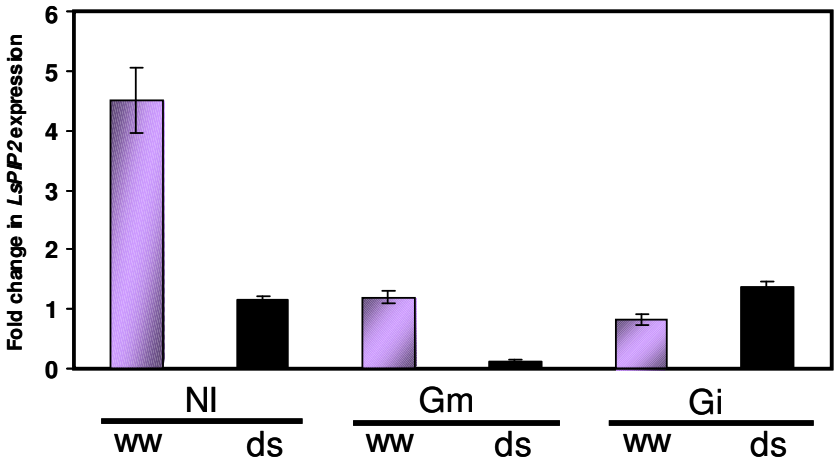

Figure 5. Fold change in *LsPIP2* gene expression determined by quantitative real-time PCR in non-inoculated control plants (NI) or in plants inoculated with *G. mosseae (Gm)* or with *G. intraradices* (Gi). Plants were either well-watered (ww) or drought stressed (ds). Data represent the mean of five replicates. Bars represent SE.

6.3.10. Western blot

We did western blot analyses on soybean and lettuce roots using PIP1 and PIP2 antibodies from *Arabidopsis thaliana*. Unfortunately, none of the antibodies recognized the corresponding protein in soybean. In lettuce, only PIP1 antibody recognized the corresponding protein, while no results were obtained for PIP2 antibody. The gene expression study has focussed more on PIP2 but, unfortunately, we only can correlate the gene expression study with data on protein levels for *LsPIP1* gene. Results in lettuce are shown in Figure 6 and paralleled results from northern blot shown in Figure 3B, demonstrating the

important decrease of PIP1 protein accumulation in lettuce plants colonized by *G. mosseae* under drought stress conditions.

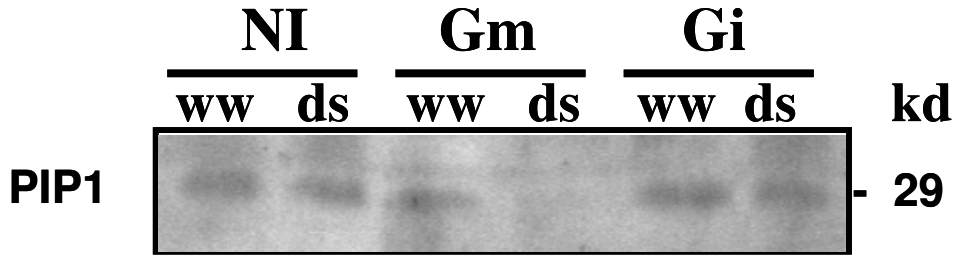

Figure 6. Western blot analysis of PIP1 protein accumulation in lettuce roots. Treatments are designed as NI, noninoculated controls; Gm, *Glomus mosseae* and Gi, *Glomus intraradices*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days.

6.4. Discussion

Tolerance to drought stress in plants is a complex phenomenon and involves many changes at both biochemical and physiological levels (Ingram and Bartels, 1996). Mechanisms of osmotic adjustment and modulation of tissue hydraulic conductivity are required to maintain tissue water potential (Bohnert *et al.,* 1995). Such mechanisms, which regulate water flux, are likely to be mediated, in part, by aquaporins (Maurel, 1997).

The discovery of aquaporins has lead to the realization that water flow across membranes may be regulated not only by osmotic pressure differences, but also by modulating the abundance and/or the activity of aquaporins (Martre *et al.,* 2002). Water that is lost from the leaves through transpiration is replenished by an apoplastic and transcellular water flow from cells that have a higher water potential. Transcellular flow requires the movement of water across the tonoplast and the plasma membrane and such transmembrane water movement is facilitated by aquaporins (Maurel, 1997; Schäffner, 1998; Luu and Maurel, 2005). It has been proposed that regulation of PIPs in the plasma membrane of root cells may play a key role in controlling radial water uptake, whereas TIPs may rather determine a general role of the vacuole in buffering osmotic fluctuations in the cytoplasm (Maurel *et al.,* 2002).

PIP aquaporins have been shown to be regulated by drought at the transcript level (Mariaux *et al.,* 1998). In this study all the genes studied showed the highest sequence homology to PIP aquaporins. We aimed to investigate if the AM symbiosis alters the pattern of PIP gene expression as a mechanism to improve plant tolerance to drought stress. Previous studies have shown that the AM fungi can take up water from soil and transfer such water to the host plant. This has been proposed as a mechanism that can help the plant to cope with drought stress (Hardie, 1985; Faber *et al.,* 1991; Ruiz-Lozano and Azcón, 1995; Marulanda *et al.,* 2003; Porcel *et al.,* 2003). If AM fungi are transferring water to the root of the host plants, it is expected that the plant must increase its permeability for water and that aquaporin genes should be up-regulated in order to allow a higher rate of transcellular water flow (Javot and Maurel, 2002). In

fact, aquaporins are enriched in zones of fast cell division and expansion, or in areas where water flow or solute flux density would be expected to be high. This included biotrophic interfaces between plants and symbiotic bacteria or fungi, as is the case of the AM symbiosis (Tyerman *et al.,* 2002).

In contrast, to the above hypothesis, our results show that the genes studied here are down-regulated both in soybean and lettuce under drought stress and that such down-regulation is even more severe in AM plants than in nonAM plants. A similar result has been obtained very recently by Ouziad *et al.* (2005) regarding the expression of PIP and TIP genes in roots of AM tomato plants subjected to salt stress. The down regulation of the aquaporin genes is not as evident in soybean plants from the first experiment, since the down-regulation of both *GmPIP* genes in AM and in noninoculated plants is of a similar magnitude (Figures 1A and 4). However, when the expression of *GmPIP2* is analyzed in a time-course, it is clearly visible that AM plants already down-regulated that gene significantly at 5 dai and 12 dai, while both nonAM control plants still maintained *GmPIP2* gene expression almost unaltered. At 20 dai, the more intense down-regulation of that gene in AM plants than in both nonAM plants was still clearly visible. Finally, at 35 days all treatments had the same level of *GmPIP2* gene expression. This effect of the AM symbiosis anticipating the down-regulation of *GmPIP2* gene may have a physiological importance to help AM plants to cope with drought stress. In fact, according to Aharon *et al.* (2003), the over-expression of a PIP aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions, but the over-expression of such PIP gene has no beneficial effect under salt stress, and has even negative effect during drought stress, causing fast wilting. Hence, the decreased expression of plasma membrane aquaporin genes during drought stress in AM plants can be a regulatory mechanism to limit the water lost from the cells (Barrieu *et al.,* 1999). In support of this hypothesis data on leaf Ψ and RWC show that AM plants (soybean and lettuce) had a less negative Ψ and also higher water content than non-AM plants.

The up- or down-regulation by drought stress of mRNAs encoding aquaporins homologues has been described in the roots of many plant species (Javot and Maurel, 2002). There are currently two opposite descriptions of the role of aquaporins in response to dehydration stress (Smart *et al.,* 2001). The first is based on evidence that expression of some aquaporins is induced under dehydration stress (Fray *et al.,* 1994; Yamada *et al.,* 1997; Barrieu *et al.,* 1999; Jang *et al.,* 2004), which is predicted to result in greater membrane water permeability and facilitated water transport. The second is based on the fact that aquaporin activity is down-regulated under dehydration stress, which should result in decreased membrane water permeability and may allow cellular water conservation (Yamada *et al.,* 1995; Johansson *et al.,* 1998; Smart *et al.,* 2001) during periods of dehydration stress.

Data obtained with lettuce plants also colonized by *G. mosseae* point in the same direction, namely that under drought stress conditions there is a higher down-regulation of the PIP genes studied (and also at the protein level, as revealed by western blot) in AM plants than in non-AM plants. In contrast to *G.*

mosseae, plants colonized by *G. intraradices* do not exhibit such down-regulation of PIP gene expression or protein accumulation. The expression of PIP genes under drought stress in these plants is similar to control non-AM plants. 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 the same way, previous studies from our research group showed a differential regulation by both AM fungi of the expression of genes encoding late embryogenesis abundant proteins (Porcel *et al.,* 2005b).

The exact reason for the different 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. *G. intraradices* seems to have an important capacity to enhance the rate of water uptake by lettuce roots. This means that the water movement in these roots must be enhanced and thus, the root water permeability must also increase, maybe by maintaining high levels of PIP aquaporin gene expression as we observe in this study. Contrarily, *G. mosseae* seems to direct its strategy for plant protection against water deficit toward the conservation of the water existing in the plant and by that reason down-regulates the expression of PIP genes. Such down-regulation of PIP genes has been interpreted as a mechanism to decrease membrane water permeability and to allow cellular water conservation (Yamada *et al.,* 1995; Johansson *et al.,* 1998; Smart *et al.,* 2001). In any case, both strategies seems to protect the host plant in a similar way since lettuce plants had similar RWC and leaf Ψ regardless of the fungus colonizing their roots.

A curious result obtained in this study concerns the reduced *GmPIP2* gene expression in Br plants under well-watered conditions. Apart from the studies describing the soybean Nodulin-26 aquaporin (Dean *et al.,* 1999; Niemietz and Tyerman, 2000), we do not know of any other study describing an effect of *Rhizobia* on the expression of aquaporins or its meaning for the biological nitrogen fixation. In any case, it is likely that this symbiosis can affect the expression of aquaporin genes as consequence of the changes that the root cells must undergo to accommodate the nodules. In addition, it must be considered that aquaporins not only are water channels but they also allow passage to small neutral molecules

such as glycerol or urea, or small gases such as ammonia or CO_2 (Luu and Maurel, 2005). Hence, it can not be discarded that the rhizobial symbiosis can be also regulating the *PIP* gene expression under well-watered conditions.

In conclusion, results from this study suggest that AM plants respond to drought stress by down-regulating the expression of the two *PIP* genes studied and anticipating its down-regulation as compared to non-AM plants, rather than by maintaining high levels of these PIP genes expression. This down-regulation 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 multi-gene family, other *PIP* isoforms in soybean and lettuce plants may be regulated differently.

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IDENTIFICACIÓN DE UN GEN DEL HONGO MICORRÍCICO ARBUSCULAR (MA) *Glomus intraradices*, QUE CODIFICA UNA PROTEÍNA 14-3-3, Y QUE RESULTA INDUCIDO POR DÉFICIT HÍDRICO DURANTE LA SIMBIOSIS MA

Resumen

El presente estudio permitió identificar un gen de *Glomus intraradices* que codifica una proteína 14-3-3, la hibridación diferencial de una genoteca de ADNc del hongo *G. intraradices* crecido *in vitro* y sometido a déficit hídrico mediante adición de PEG 6000 al 25%. Posteriormente se procedió a estudiar el patrón de expresión de este gen fúngico bajo condiciones de estrés hídrico *in vitro* y cuando estaba formando una simbiosis natural con diferentes plantas hospedadoras. Los resultados obtenidos sugieren que el gen *Gi14-3-3* puede estar implicado en la protección que la simbiosis MA confiere a la planta hospedadora frente a la sequía. Estos hallazgos han proporcionado nuevas evidencias de que la contribución de los hongos MA a la mejora de la tolerancia a la sequía en la planta hospedadora puede estar mediada por un grupo de proteínas (las 14-3-3) que regulan tanto rutas de señalización metabólicas como las proteínas efectoras implicadas en la respuesta final de la planta ante un estímulo ambiental.

Palabras clave: simbiosis micorrícico arbuscular, proteína 14-3-3, estrés hídrico

IDENTIFICATION OF A GENE FROM THE ARBUSCULAR MYCORRHIZAL (AM) FUNGUS *Glomus intraradices* ENCODING FOR A 14-3-3 PROTEIN, THAT IS UP-REGULATED BY DROUGHT STRESS DURING THE AM SYMBIOSIS

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 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 signalling pathways and also effectors proteins involved in the final plant responses.

Keywords: *Arbuscular mycorrhizal symbiosis, 14-3-3 protein, drought stress*

7.1. 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 signalling 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 detection of interactions between 14-3-3's and proteins with signalling or protective functions [32; 43; 58].

Many studies on 14-3-3 proteins have been carried out in plants, animals and yeasts. 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 non-targeted 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.

7.2. Materials and Methods

7.2.1. 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 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 five days and then harvested and stored in liquid nitrogen for subsequent RNA extraction, library construction and RT-PCR experiments.

7.2.2. 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 3 consecutive days). 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.

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 *Nicotiana tabaccum* were used [38; 54]. All plants were inoculated with the AM fungus *Glomus 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), while the other half was drought-stressed for 10 days before harvest (ds) as previously described [36; 38; 39].

7.2.3. 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 (two 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 24h-period comprised between each rewatering the soil water content was progressively decreasing until a minimum value of 60% of field capacity.

7.2.4. 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].

7.2.5. 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, California) 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 λ bacteriophage particles were plated at low density (1500-2000 pfu per plate) on the host *E. coli* (strain NM522). After incubation for 7 to 8 h at 37 °C, the colonies were produced and the petri plates placed at 4 °C for 1h to harden the top agarose. They were then overlaid with a Hybond-N^+ nylon membrane (Amersham, Little Chalfont, UK) 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 were 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 2X SSC and 0.1% SDS, and once for 15 min at 65 °C in 0.5X 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 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 [29]. 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 vitro* and added or not added of 25% PEG 6000.

7.2.6. Sequencing the cloned cDNA and analyses.

Sequencing was performed by the dideoxy-sequencing method [52] 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).

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

7.2.7. 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, Little Chalfont, UK) by capillarity [51]. Equal RNA loading and transfer were verified by methylene blue staining of nylon membranes before hydridization [22]. Blots were prehybridized 2-3 h at 65 °C in 5X Denhardt's solution, 5X SSC, 0.5% SDS and hybridized with *Gi14-3-3* specific probe obtained by radioactive PCR labelling of plasmid insert. Unincorporated ^{32}P was removed using Mini Quick SpinTM 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 2X SSC and 0.1% SDS, and twice for 15 min at 65 °C with 0.5X 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 (BioRad, 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.

7.2.8. Quantitative real time RT-PCR

Gi14-3-3 gene expression was studied by real-time PCR by using iCycler (Bio-Rad, Hercules, California, USA), as described by González-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, 1x first strand buffer (Invitrogen, Carlsbad, California, 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

tgttaataaaaatcggtgcgttgc; 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 1x SyBR Green (Molecular Probes, Eugene, Oregon, USA), and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) in 1x PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl).

The PCR program consisted in 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 procedure was checked with a heat dissociation protocol (from 70 °C 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.

7.2.9. Nucleotide sequence accession number

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

7.3. Results

7.3.1. Symbiotic development

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

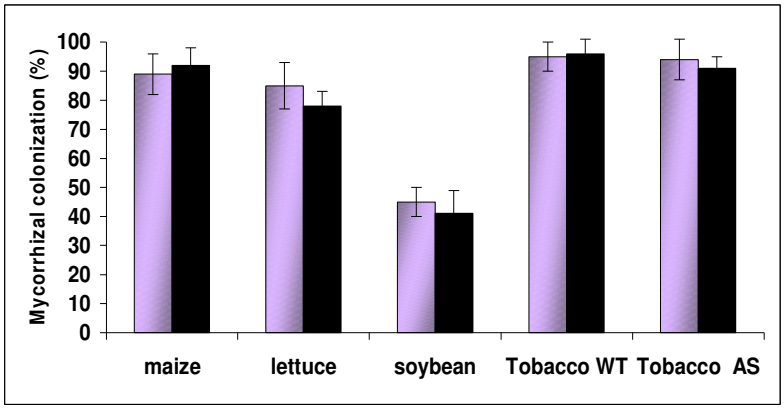

Figure 1. Percentage of mycorrhizal root infection by *G. intraradices* in the different plants used in this study. Plants were either well-watered (coloured columns) or drought stressed for 10 days (black columns). Data are means of four replicates and lines over the columns represent the standard error.

7.3.2. 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 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' nontraslated 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].

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

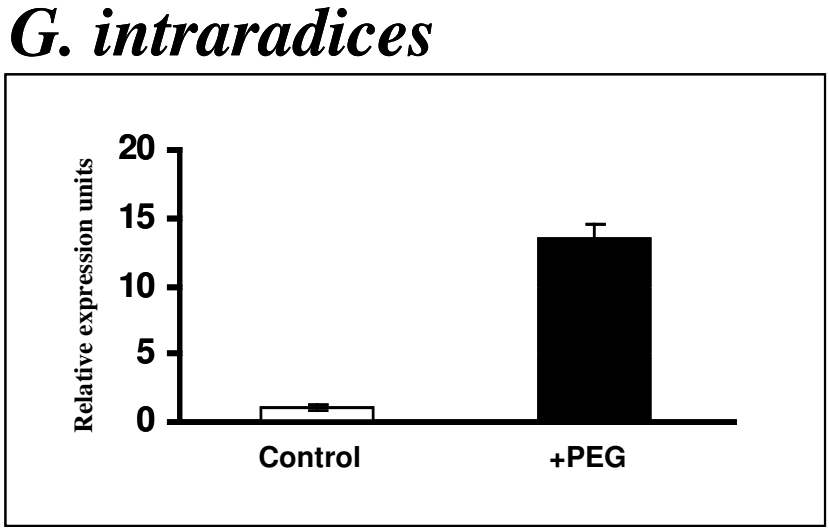

Figure 2. Analysis of *Gi14-3-3* gene expression by real time quantitative RT-PCR in the AM fungus *G. intraradices* grown *in vitro*. The fungus was subjected to drought by addition of PEG (25%) to the growing medium or maintained under control conditions without PEG.

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, (Figure 3).

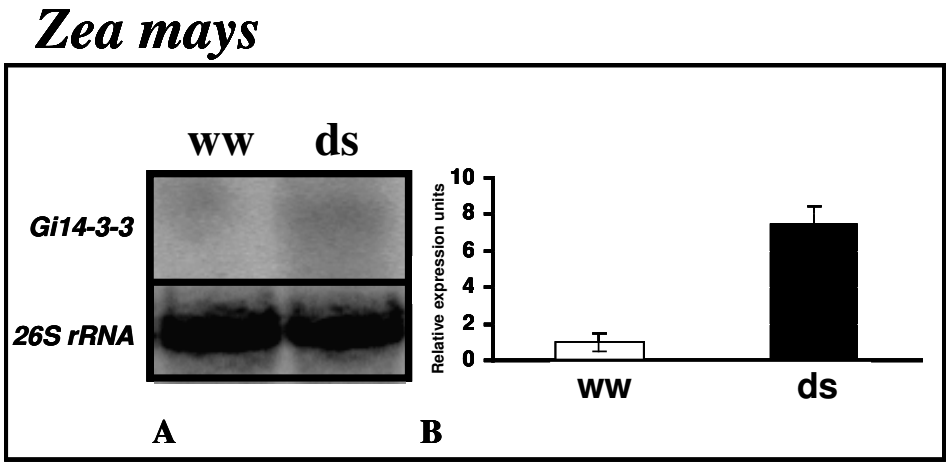

Figure 3. Analysis of *Gi14-3-3* gene expression in maize plants colonized by the AM fungus *G. intraradices*. Plants were either well-watered (ww) or drought stressed (ds). (**A**) northern blot of total RNA (15 μg). The lower panels show the amount of 26S rRNA loaded for each treatment (methylene blue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

A lower increase (38%) in gene expression was observed in lettuce plants subjected to drought stress (Figure 4), while in tobacco (Figure 5) the increase of *Gi14-3-3* gene expression was by 823% and 127% for wildtype and antisense plants, respectively.

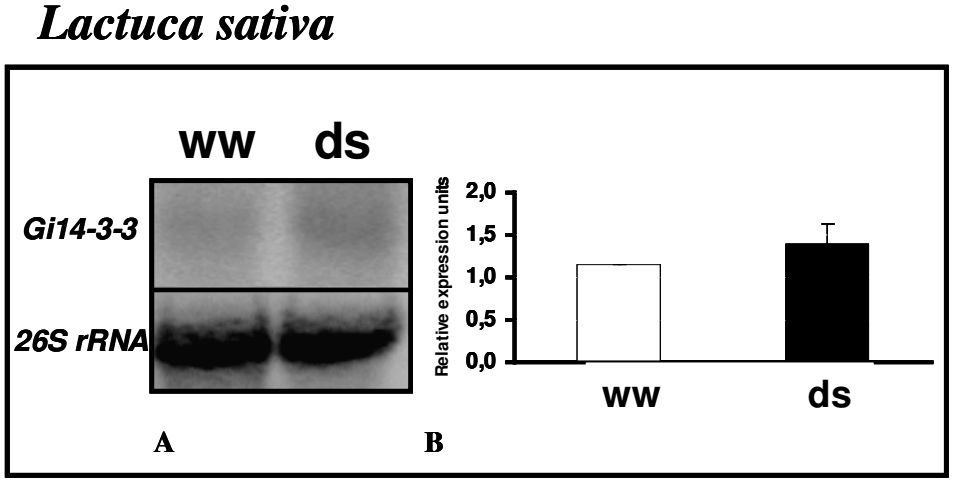

Figure 4. Analysis of *Gi14-3-3* gene expression in lettuce plants colonized by the AM fungus *G. intraradices*. See legend for Figure 3.

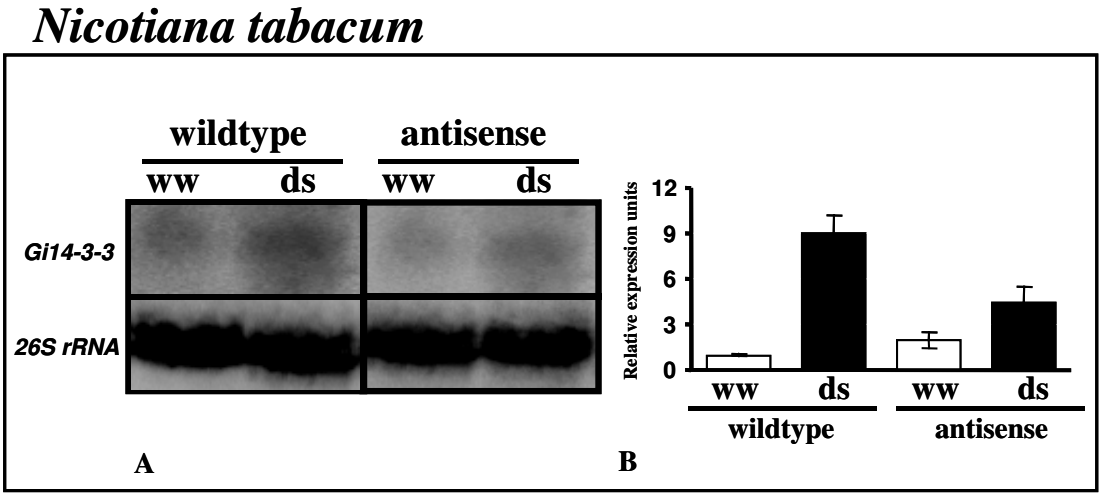

Figure 5. Analysis of *Gi14-3-3* gene expression in two tobacco plant lines (wildtype or an aquaporin antisense mutant) colonized by the AM fungus *G. intraradices*. See legend for Figure 3.

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 (Figure 6).

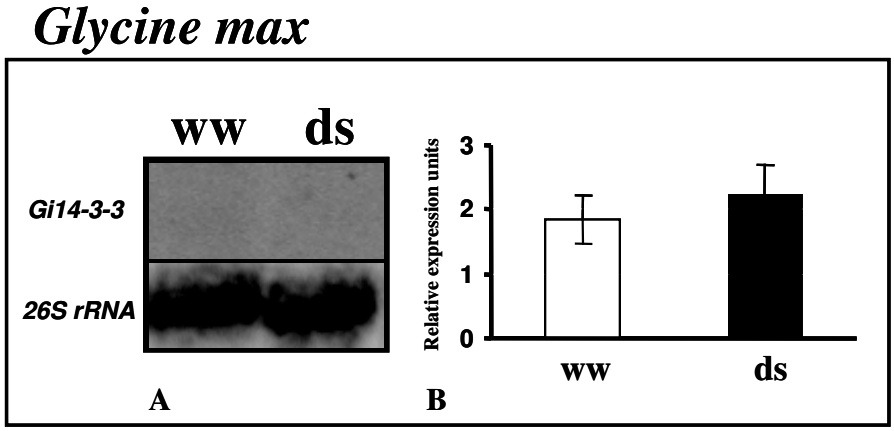

Figure 6. Analysis of *Gi14-3-3* gene expression in soybean plants colonized by the AM fungus *G. intraradices*. See legend for Figure 3.

7.3.4. 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 (Figure 7).

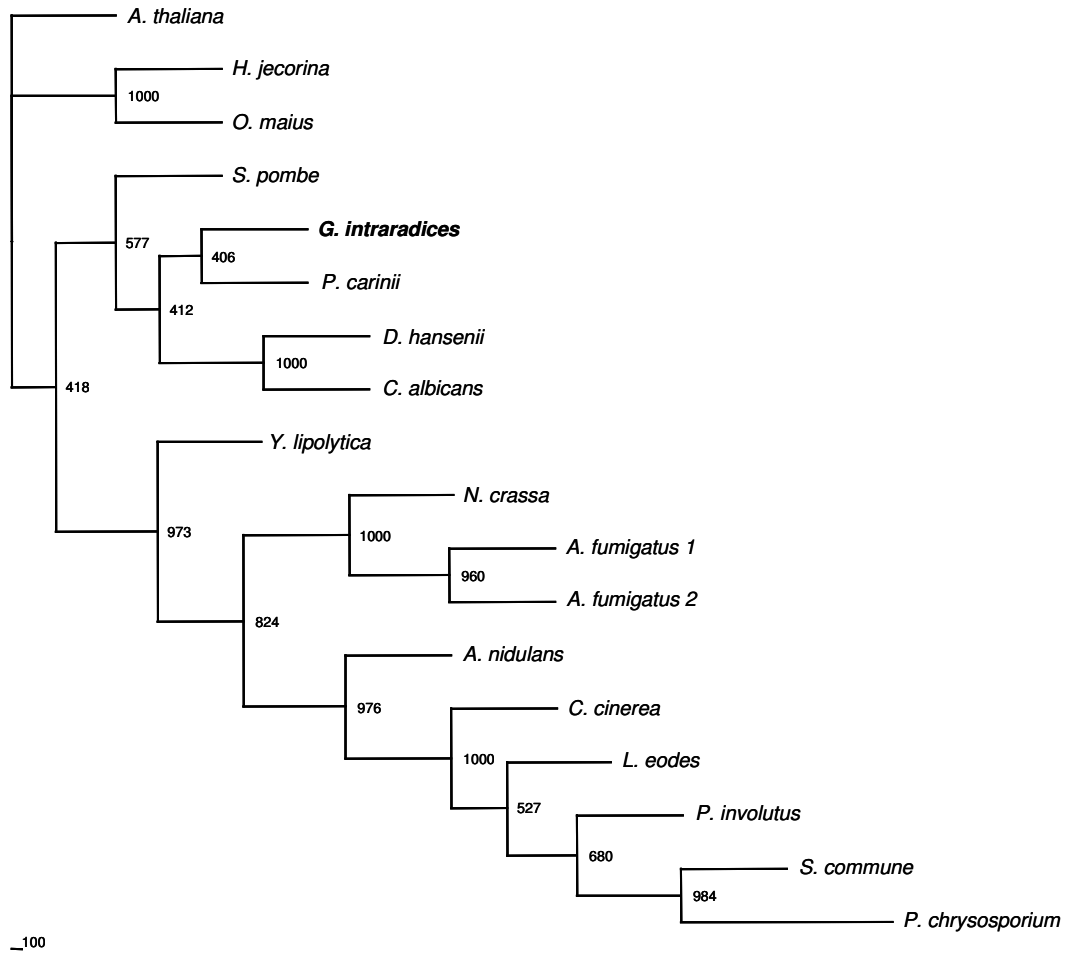

Figure 7. Phylogenetic tree constructed after alignment of *Gi14-3-3* sequence from *G. intraradices* and other fungi. The tree was constructed using the Neighbour-Joining method from the program package Phylip. Scale represents the estimated number of nucleotide substitutions per sequence position. Accession numbers of sequences used to construct the three are as follows: *Arabidopsis thaliana* NM202155, that was set up as the outgroup in the three; *Aspergillus fumigatus* 1 NW876235; *Aspergillus 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.

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.

7.4. Discussion

Abiotic stresses, such as drought, salinity or extreme temperatures share a common osmotic component due to 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 in order to prevent cellular damage and to re-establish 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 localisation 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 cCNA library aimed at identifying such induces genes. 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 since 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 arbuscular mycorrhizal 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.

As well as 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 consitute a unique family of plant kinases which are defined by a C-terminal calmodulin-like regulatory domain and have been shown to be involved in drought and salinity responses [15]. 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 plants lines that exhibited a higher induction of *Gi14-3-3* gene expression in wildtype plants and a lower induction in the plant line that is more sensitive to drought (antisense). Antisense tobacco plants show an 80% of 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 Gi-14-3-3 protein can regulate the activity of plasma membrane H+-ATPases of either the fungus or the host plant, in order to activate its pumping activity, which is essential to cope with osmotic stress [11; 33; 53]. Additionally, Gi-14-3-3 protein can activate a fungal or plant

calcium-dependent protein kinase in order to modulate the activity of final effectors 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 signalling pathways and also proteins involved in the final responses.

7.5. Acknowledgement

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UN GEN DEL HONGO MICORRÍCICO ARBUSCULAR (MA) *Glomus intraradices* QUE CODIFICA UNA PROTEÍNA DE UNIÓN RESULTA INDUCIDO POR SEQUÍA DURANTE LA SIMBIOSIS MICORRÍCICA

Resumen

La contribución de la simbiosis micorrícico arbuscular (MA) a la tolerancia de las plantas a la sequía da lugar a una combinación de efectos físicos, nutricionales y celulares. Sin embargo, los mecanismos exactos implicados en esta mejora son todavía un debate. En este estudio se ha identificado un gen del hongo MA *Glomus intraradices* que codifica para BiP, mediante hibridación diferencial de una genoteca de ADNc del hongo crecido *in vitro* y sometido a déficit hídrico por adición de 25% PEG 6000. Los resultados mostraron que su expresión estaba inducida por déficit hídrico no sólo durante las condiciones *in vitro* (cultivo monoxénico MA) sino también *ex vitro*, cuando formaba simbiosis natural con plantas. La identificación del gen *GiBiP* ha proporcionado evidencias de que la contribución de los hongos MA a la mayor tolerancia de las plantas hospedadoras frente a la sequía puede estar mediada por proteínas con actividad chaperona, como la de las proteínas BiP.

Palabras clave: simbiosis micorrícico arbuscular, BiP, déficit hídrico

A GENE FROM THE ARBUSCULAR MYCORRHIZAL FUNGUS *Glomus intraradices* ENCODING A BINDING PROTEIN IS UP-REGULATED BY DROUGHT STRESS DURING THE MYCORRHIZAL SYMBIOSIS

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*

8.1. Introduction

Water deficit is one of the most common environmental stress factors experienced by soil 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 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 *et al.,* 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, 1999). 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, 1999). 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. 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, 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. 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.

8.2. Materials and Methods

8.2.1. 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. 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 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 five days and then harvested and stored in liquid nitrogen.

8.2.2. 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*) and tobacco (*Nicotiana tabacum*). Two plant lines (an aquaporin antisense mutant and the corresponding wildtype) from *Nicotiana tabaccum* were used (see Porcel *et al.,* 2005a; *Siefrizt et al.,* 2002). All plants were inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) isolate BEG 121. 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).

8.2.3. 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 (two 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 24h-period comprised between each rewatering the soil water content was progressively decreasing until a minimum value of 60% of field capacity.

8.2.4. 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).

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

Total RNA was extracted from *G. intraradices* mycelia grown in monoxenic culture under control 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, California) 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.

8.2.6. 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).

8.2.7. 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 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.

8.2.8. Quantitative real time RT-PCR

GiBiP gene expression was studied by real-time PCR by using iCycler (Bio-Rad, Hercules, California, USA), as described by González-Guerrero *et al.* (2005). The primer set used to amplify *GiBiP* and *Gi 18S rRNA* genes in the synthesized cDNAs were the following (5' to 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 $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Negative controls without cDNA were used in all PCR reactions.

8.3. Results

8.3.1. Symbiotic development

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

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

Table 1. Percentage of mycorrhizal root infection by *G. intraradices* in the different plants used in this study. Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple-range test ($n = 4$).

8.3.2. Isolation 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 AJ319773, corresponding to the *BiP* gene from *G. intraradices*.

8.3.3. 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 (Figure 1). The addition of PEG to the medium increased *GiBiP* gene expression by 41%.

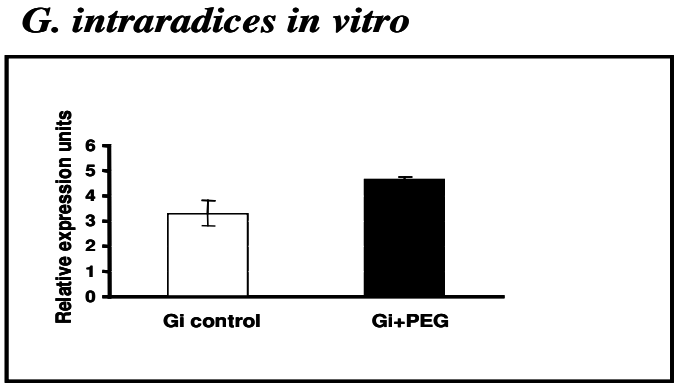

Figura 1. Analysis of *GiBiP* gene expression by real time quantitative RT-PCR in the AM fungus *G. intraradices* grown *in vitro*. The fungus was subjected to drought by addition of PEG 6000 (25%) to the growing medium or maintained under control conditions without PEG.

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, (Figure 2).

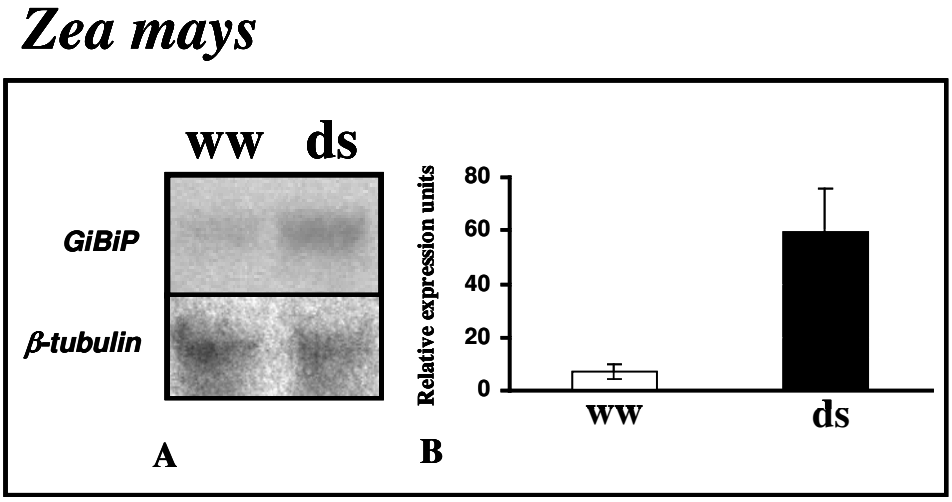

Figure 2. Analysis of *GiBiP* gene expression in maize plants colonized by the AM fungus *G. intraradices*. Plants were either well-watered (ww) or drought stressed (ds). (A) northern blot of total RNA (15 μg). Blots were hybridized with *GiBiP* probe and with the *G. intraradices* β-tubulin probe (Accession BE603903) for calibration of *GiBiP* gene expression. (B) real time quantitative RT-PCR analysis of *GiBiP* gene expression.

A lower increase (52%) in gene expression was observed in soybean plants subjected to drought stress (Figure 3) and also in wildtype tobacco plants (Figure 4), that increased by 106% *GiBiP* gene expression.

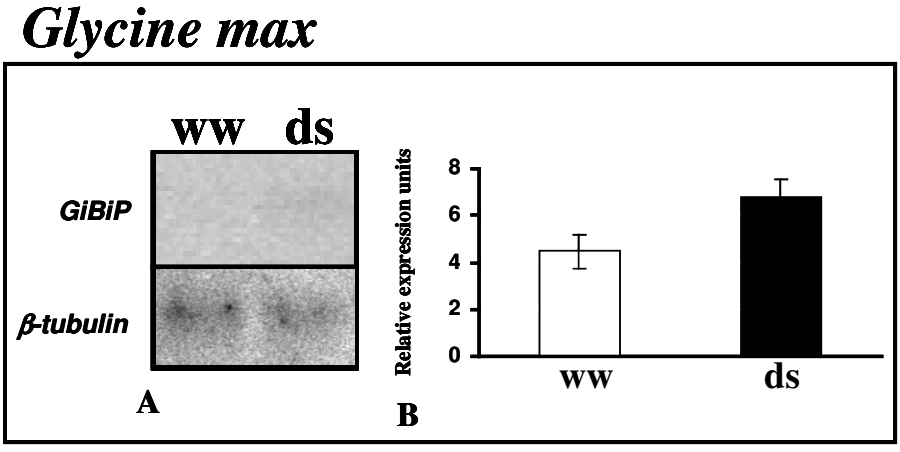

Figure 3. Analysis of *GiBiP* gene expression in soybean plants colonized by the AM fungus *G. intraradices*. See legend for Figure 2.

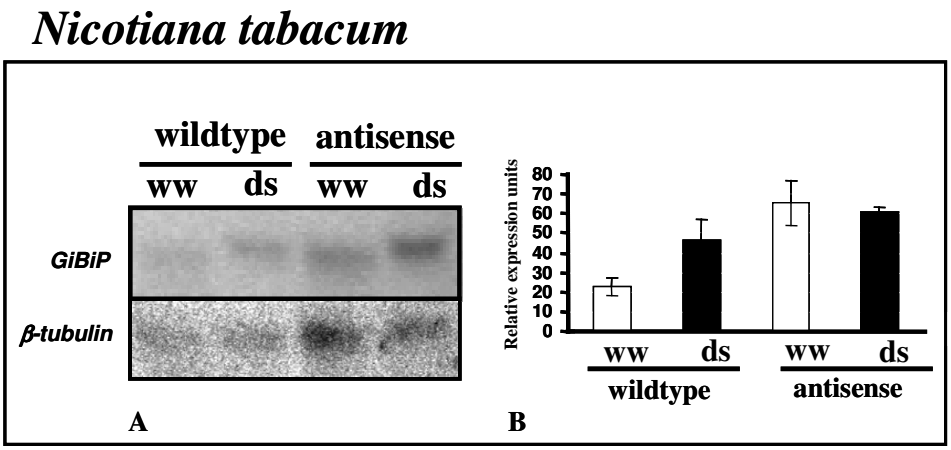

Figure 4. Analysis of *GiBiP* gene expression in two tobacco plant lines (wildtype or an aquaporin antisense mutant) colonized by the AM fungus *G. intraradices*. See legend for Figure 2.

In contrast, when the host plant was the antisense tobacco line or lettuce, *GiBiP* gene expression was not significantly affected by drought stress (Figures 4 and 5).

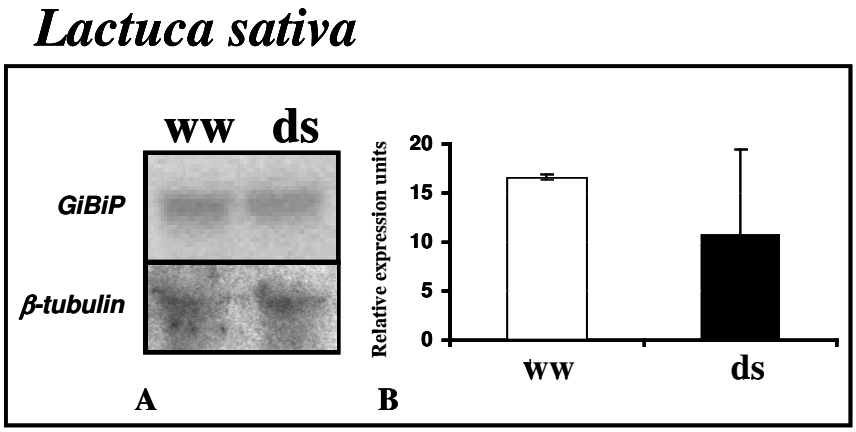

Figure 5. Analysis of *GiBiP* gene expression in lettuce plants colonized by the AM fungus *G. intraradices*. See legend for Figure 2.

8.3.4. 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 gene has a role in the answer of the fungus against drought. This gene 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). 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 showed that the protection the AM symbiosis confers to these plants against drought stress, evidenced in several former studies (Ruiz-Lozano, 2003; Porcel *et al.,* 2005a), is not mediated by BiP activity. This protection should be mediated by other mechanisms, as those already reported in previous revisions on the subject (Augé, 2001, Ruiz-Lozano, 2003).

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. 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* 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; Ricardi *et al.,* 1998) and BiP has been shown to associate with water-stress induced proteins (Cascardo *et al.,* 2000). Our findings provide 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.

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8.3.6. References

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A nivel mundial, la falta de agua es el factor limitante más severo para el crecimiento y la productividad de las plantas (Kramer y Boyer 1997), y esto es especialmente palpable en los ecosistemas mediterráneos. Se estima que alrededor de un 28% de los suelos terrestres están demasiado secos para soportar un correcto desarrollo de las plantas (Bray, 2004), de manera que la supervivencia y el rendimiento de las cosechas se ve negativamente afectada por el déficit hídrico. Afortunadamente, las plantas no crecen solas, sino que lo hacen asociadas a numerosos microorganismos del suelo que interaccionan con ellas, dando lugar a diversas acciones que producen beneficios para las plantas (Jeffries y Barea, 1994; Barea, 1998). Entre estos microorganismos destacan los hongos formadores de MA. El papel ecofisiológico de las MA no se limita a su participación en la captación y transferencia de nutrientes hacia la planta hospedadora, sino que se han descrito otros muchos efectos beneficiosos para la planta y para el ecosistema. Por lo tanto, se considera que las MA desempeñan un papel crucial en la supervivencia y desarrollo de las plantas, sobre todo en suelos sometidos a condiciones de estrés como sequía, salinidad, cambios bruscos de temperatura o deficiencia de nutrientes (Sánchez-Díaz y Honrubia, 1994; Azcón-Aguilar y Barea, 1996; Ruiz-Lozano, 2003). En cualquier caso, los mecanismos exactos por los que se consigue este efecto protector no son aún del todo conocidos. Este trabajo de Tesis Doctoral pretende contribuir precisamente a incrementar el conocimiento acerca de los mecanismos por los cuales la simbiosis MA incrementa la tolerancia de las plantas frente al déficit hídrico.

El estudio se inició evaluando la contribución de la simbiosis MA en la protección de las leguminosas frente al daño en el funcionamiento de los nódulos fijadores de N_2 producido por la sequía. Las plantas de la familia *Leguminoseae* pueden formar una doble simbiosis con bacterias fijadoras de N_2 de la familia *Rhizobiaceae*, así como con hongos formadores de MA. La simbiosis con los rhizobios proporciona a la planta el N necesario para su crecimiento, mientras que la importancia de la simbiosis MA en las leguminosas se ha atribuido tradicionalmente a los altos requerimientos en P para los procesos de nodulación y fijación de N_2 (Barea y Azcón-Aguilar, 1983). No obstante, el papel de los hongos MA en esta simbiosis tripartita parece no limitarse a este efecto meramente nutricional (Barea *et al.*, 1988; Goicoechea *et al.*, 1997). En efecto, el déficit hídrico tiene un impacto negativo considerable en el funcionamiento de los nódulos de leguminosas (Sprent, 1971). Entre otros efectos perniciosos, inhibe la fotosíntesis de la planta y afecta al delicado mecanismo de control del oxigeno en los nódulos, que son procesos esenciales para que se pueda dar una fijación de nitrógeno activa (Sprent, 1971; Becana *et al.*, 1986; Sprent y Zahran, 1988; Irigoyen *et al.*, 1992; Gogorcena *et al.*, 1995), desencadenando así el proceso de senescencia nodular prematura. Un estudio realizado en nuestro laboratorio ha puesto de manifiesto un efecto protector de la simbiosis MA frente a la senescencia nodular prematura inducida por el déficit hídrico (Ruiz-Lozano *et al.*, 2001b). En este estudio se comprobó que la simbiosis MA incrementó el peso seco

de los nódulos en comparación con las plantas no micorrizadas y aumentó la actividad nodular (medida como actividad acetileno reductasa, ARA) en un 112%, el contenido en leghemoglobina de los nódulos en un 25% y el de proteínas en un 15%. En cualquier caso, los resultados más interesantes se obtuvieron en relación con el daño oxidativo a biomoléculas como lípidos y proteínas, que resultó incrementado por el déficit hídrico en todos los tratamientos, pero fue considerablemente menor en los nódulos de las plantas MA que en las plantas no micorrizadas. Es por ello que se propuso que el mecanismo principal por el cual la simbiosis MA había protegido a las plantas de soja frente al fenómeno de senescencia nodular prematura era mediante una disminución del daño oxidativo a biomoléculas (Ruiz-Lozano *et al.*, 2001b).

Con estos antecedentes, el siguiente paso en esta investigación sobre la protección de la simbiosis MA frente a la senescencia nodular prematura inducida por sequía en leguminosas debía estar, por tanto, encaminado a determinar las causas de ese menor daño oxidativo a biomoléculas en las plantas micorrizadas. Así es como se inicia el presente trabajo de Tesis Doctoral presentado en el capítulo primero de esta memoria. De hecho, el menor daño oxidativo en los nódulos de las plantas micorrizadas se podía deber bien a que esas plantas hubieran sufrido menos el déficit hídrico que las plantas no micorrizadas debido a mecanismos de evitación primaria del estrés (por ejemplo mediante captación directa de agua por las hifas fúngicas y transferencia a la planta hospedadora), lo que las habría protegido de la generación de EROs, o bien a que las plantas micorrizadas hubieran incrementado su nivel de protección frente a EROs mediante la inducción de actividades antioxidantes. Los resultados obtenidos en relación a la primera hipótesis indican que, en condiciones de déficit hídrico, las plantas MA fueron capaces de extraer diariamente más agua del suelo que las no micorrizadas, lo que está de acuerdo con estudios previos que sugieren una mayor capacidad para retener agua en los suelos donde crecen plantas micorrizadas (Augé *et al.*, 2001, 2004) y también que las hifas fúngicas pueden extraer agua desde fuentes no accesibles para la raíz y transferirla a la planta hospedadora (Hardie, 1985; Ruiz-Lozano y Azcón, 1995; Marulanda *et al.*, 2003).

El análisis de cuatro actividades antioxidantes como SOD, CAT, APX y GR demostró que sólo la actividad GR resultó consistentemente incrementada por la simbiosis MA tanto en raíces de soja como en los nódulos. La GR es el enzima que regenera el glutation oxidato hasta su forma reducida en el ciclo del ascorbato-glutation, siendo el glutation (GSH) el tiol de bajo peso molecular más abundante en todos los seres vivos (Foyer *et al.*, 1991). El GSH se encuentra en concentraciones milimolares en diferentes tejidos donde, generalmente, actúa como agente reductor. Además tiene otras funciones importantes como la de eliminar EROs en el ciclo del ascorbato-glutation o la inducción de enzymas, y participa en el metabolismo del azufre y en la expresión de varios genes (Foyer *et al.*, 1995). Por lo tanto, este incremento de actividad GR en plantas MA puede ser de importancia en relación con la disminución del daño causado por el déficit hídrico en las plantas de soja. De hecho, Kranner (2002) relacionó la cantidad de GSH reducido con los diferentes grados de tolerancia a la desecación en líquenes y

se ha propuesto que la actividad GR juega un importante papel en la protección de las plantas frente a varias formas de estrés (Aono *et al.*, 1995). Así, los resultados de este estudio sugieren que los niveles consistentemente mayores de actividad GR en las raíces y nódulos de las plantas MA pueden haber contribuido a generar antioxidantes reducidos (GSH) que ayudarían a disminuir el daño oxidativo a biomoléculas responsable, en última instancia, de la senescencia nodular prematura (Becana *et al.*, 2000).

Así pues, la simbiosis MA protegería a las plantas de soja frente al fenómeno de la senescencia nodular prematura inducida por sequía mediante una combinación de mejor explotación de los recursos hídricos de la planta y de incremento de las defensas antioxidantes con el consiguiente menor daño oxidativo a biomoléculas. Para estudiar más en profundidad la repercusión de la mejor explotación de los recursos hídricos de la planta se planteó la investigación recogida en el capítulo segundo de esta memoria. Se trataba de evaluar los efectos de la simbiosis MA sobre el estatus hídrico de las plantas de soja en condiciones de déficit hídrico, con especial énfasis en parámetros como el potencial hídrico o el ajuste osmótico, y sus repercusiones sobre el estrés oxidativo en estas plantas. Los resultados obtenidos en este estudio muestran que las plantas MA presentaron una mayor tolerancia al déficit hídrico impuesto. Así, en condiciones de déficit hídrico las plantas MA crecieron un 27% más que las controles sin micorrizar o presentaron un potencial hídrico foliar significativamente mayor (menos negativo) así como una reducción del 55% del daño oxidativo a lípidos en hojas. En relación con la osmoregulación, la acumulación de azúcares solubles en hoja fue menor en las plantas MA sometidas a déficit hídrico que en las plantas control sin micorrizar. Este hecho fue observado también por Schellembaum *et al.* (1998), quienes sugirieron que se podía deber a que los hongos MA pueden resultar unos competidores importantes de fuentes carbonadas dirigidas hacia la raíz cuando las condiciones de cultivo limitan la fotosíntesis, como es el caso del déficit hídrico. Sin embargo, la prolina también se acumuló menos en las hojas de las plantas MA que en las de las plantas control, lo que sugiere que realmente estas plantas sufrieron en menor medida los efectos del déficit hídrico debido a una evitación primaria del mismo (Subramanian *et al.*, 1995), como lo demuestra los valores más altos de potencial hídrico en hojas de las plantas MA (-1.9 MPa) que en las plantas sin micorrizar (-2.5 MPa). Curiosamente, en raíz, la prolina si se acumuló más en las plantas MA que en las plantas control sin micorrizar, de manera que proponemos que la simbiosis MA primero aumenta el ajuste osmótico en la raíz de la planta hospedadora, lo que contribuye a mantener un gradiente de potencial hídrico favorable a la entrada de agua desde el suelo hasta la raíz de la planta. Esto permite a las plantas MA mantener un mayor potencial hídrico en sus tejidos durante los periodos de déficit hídrico y protege a estas plantas frente al estrés oxidativo, de manera que la acumulación de estos efectos repercute en la mayor tolerancia de las plantas MA frente al déficit hídrico.

Es bien sabido que la sequía induce una gran variedad de respuestas fisiológicas y bioquímicas en las plantas, de manera que hay una gran variedad de genes de la planta cuya expresión se ha demostrado varía en respuesta a la sequía

(Shinozaki y Yamaguchi-Shinozaki, 1997; Bray, 1997; 2004). Los productos de los genes inducidos por este tipo de estreses se clasifican en dos grupos: los que directamente protegen contra los estreses medioambientales y los que regulan la expresión génica y la transducción de señales en la respuesta al estrés (Shinozaki y Yamaguchi-Shinozaki, 1997). En este trabajo de Tesis Doctoral, además del estudio a nivel bioquímico que se acaba de discutir anteriormente, también se ha evaluado si la simbiosis MA altera la expresión de una serie de genes de la planta que se ha demostrado están implicados en la respuesta de la misma frente al déficit hídrico. Entre estos genes hemos seleccionado aquellos que codifican un grupo de proteínas LEA (del inglés late embryogenesis abundant), las dehidrinas (Close, 1996). Así mismo, hemos estudiado el gen que codifica P5CS, enzima implicada en la biosíntesis del osmoregulador prolina (Hu *et al.*, 1992) y también genes que codifican aquaporinas, que en plantas regulan el flujo transcelular de agua (Martre *et al.*, 2002; Luu y Maurel, 2005).

Además de una aproximación experimental dirigida hacia el estudio de genes específicos *(LEA, P5CS* y *aquaporinas*), también se ha seguido una aproximación experimental no dirigida y consistente en la construcción de una genoteca de ADNc del hongo MA *G. intraradices* crecido en cultivo monoxenico y sometido a déficit hídrico mediante la adición de PEG 6000 al medio de cultivo líquido. El análisis diferencial de esta genoteca nos ha permitido identificar dos genes fúngicos que codifican una proteína 14-3-3 y una proteína BiP, respectivamente, en *G. intraradices*, que han sido objeto de estudio igualmente.

Este apartado de la Tesis se inició evaluando el efecto de la simbiosis MA y del déficit hídrico sobre la expresión de los genes *lea* y *p5cs* de soja y lechuga. El interés por los genes *lea* viene dado por la existencia de numerosas evidencias que demuestran que las dehidrinas (grupo D11 de proteínas LEA) son el grupo de proteínas que más frecuentemente se acumulan en plantas bajo situaciones de déficit hídrico (Close, 1997; Cellier *et al.*, 1998). Parece que las dehidrinas juegan un papel fundamental en la respuesta de deshidratación de las plantas frente a un rango de estímulos ambientales (Close, 1996). La existencia de dianas múltiples para las proteínas LEA (cromatina, citosol, citoesqueleto) sugiere que las consecuencias de su actividad son muy amplias desde un punto de vista bioquímico. Así, se ha demostrado que durante la deshidratación de las células vegetales las proteínas LEA juegan un importante papel en el mantenimiento de las estructuras de otras proteínas, vesículas, membranosas, en el secuestro de iones como el calcio o en la unión al agua (Close, 1996; Heyen *et al.*, 2002; Koag *et al.*, 2003).

En cuanto a los genes *p5cs*, el interés por su estudio radica en que la proteína P5CS cataliza el paso fundamental y limitante en la biosíntesis de prolina (Hu *et al*, 1992; Kishor *et al.*, 1995), uno de los solutos compatibles más utilizados por las plantas para los procesos de osmoregulación bajo condiciones de déficit hídrico (Morgan, 1984; Kishor *et al.*, 1995). En general, la sobre-expresión de los genes que codifican enzimas implicadas en la síntesis de osmolitos aumenta la tolerancia al estrés osmótico. El aumento de la tolerancia al estrés podría deberse a que la acumulación de los osmolitos y la consiguiente disminución del potencial osmótico, permite un aumento en la absorción del agua y el restablecimiento de la

concentración intracelular de sales. Sin embargo, las manipulaciones genéticas de plantas para la producción de solutos compatibles no siempre producen una acumulación del compuesto lo suficientemente elevada como para justificar un ajuste osmótico celular lo que sugiere que la función de estas sustancias no se restringe exclusivamente al balance osmótico (Chen y Murata, 2002). Aparte de su participación en el equilibrio osmótico, se han asignado otras funciones para estas sustancias que permiten explicar el aumento de la tolerancia al estrés provocado por su acumulación a nivel celular. Entre estos posibles mecanismos de acción, se encuentran: la inducción de cambios en el potencial de membrana y en la permeabilidad de la misma para favorecer el transporte de diferentes iones, la eliminación de radicales libres y la actuación como chaperonas al estabilizar directamente membranas y/o proteínas (Lee *et al.*, 1997; Hare *et al.*, 1998; Bohnert y Shen, 1999; Zhu, 2001; Apse y Blumwald, 2002; Rai, 2002).

Los resultados obtenidos con los genes *lea* y *p5cs* estudiados muestran que ambos tipos de genes presentaron un comportamiento similar. Así, excepto en las plantas inoculadas sólo con *B. japonicum*, ambos tipos de genes se indujeron por déficit hídrico en comparación con el mismo tratamiento en condiciones óptimas, indicando que, como se esperaba, ambos tipos de genes son importantes en la respuesta de la planta frente al déficit hídrico (Colmenero-Flores *et al.*, 1997; Yoshiba *et al.*, 1997; Cellier *et al.*, 1998; Giordani *et al.*, 1999; Hare *et al.*, 2003). Curiosamente, las plantas inoculadas sólo con *B. japonicum* (Br) mostraron altos niveles de expresión de los genes *lea* en condiciones optimas de riego y no inducción de estos genes ni tampoco del gen *p5cs* a consecuencia del estrés. Para entender estos resultados divergentes respecto al resto de tratamientos es necesario tener en cuenta que la expresión tanto de genes *lea* como *p5cs* está regulada por dos tipos de rutas: una dependiente de ABA que responde a los niveles de esta hormona en la planta y otra independiente de ABA que responde al estatus hídrico de la planta, y que ambas rutas pueden actuar simultáneamente y tener efectos acumulativos (Skriver y Mundi, 1990; Chandler y Robertson, 1994; Giordani *et al.,* 1999; Ábrahám *et al.,* 2003). Así la ruta dependiente de ABA puede explicar el que las plantas Br presentaran altos niveles de expresión de este gen incluso en condiciones óptimas de riego pues se ha visto que el proceso de nodulación en leguminosas incrementa los niveles de cuatro fitohormonas, incluyendo el ABA (Hirsch y Fang, 1994), por lo tanto, el efecto observado puede ser sólo una respuesta inespecífica de los genes *lea* a un aumento del ABA en las raíces de plantas Br.

En cualquier caso, un efecto consistentemente observado en este estudio tanto con plantas de soja como de lechuga es que la expresión tanto de los genes *lea* como de los *p5cs* resultó inhibida por la simbiosis MA en las plantas sometidas a déficit hídrico (resultado contrario a nuestra hipótesis de trabajo inicial, que esperaba una inducción de estos genes por la simbiosis MA). Este efecto puede estar relacionado con la ruta dependiente de ABA que regula la expresión de estos genes ya que se ha observado que la micorrización puede disminuir los niveles de ABA en las plantas colonizadas en situaciones de déficit hídrico (Duan *et al.*, 1996; Goicoechea *et al.*, 1997; Ludwig-Müller, 2000; Estrada-Luna y Davies, 2003) lo que

redundaría en una menor inducción de estos genes en las plantas MA. De igual modo, la ruta independiente de ABA también puede haber influido en esta menor expresión de los genes *lea* y *p5cs* en las plantas micorrizadas sometidas a estrés. Así, hay numerosas evidencias de que las plantas MA sufren menos el déficit hídrico que las no MA debido a mecanismos de evitación primaria del estrés que permiten a las plantas MA mantener un mejor estatus hídrico bajo condiciones de estrés (Revisado por Augé, 2001; Ruiz-Lozano, 2003) y ello conduciría a una menor expresión de los genes *lea* y *p5cs*. De hecho, los datos del contenido hídrico relativo (CHR) de este estudio o los de Ψ en hoja del segundo capitulo de esta Tesis apuntan en este sentido, pues son significativamente más altos en las plantas MA sometidas a déficit hídrico que en las plantas no MA, indicando que su estatus hídrico era mejor.

Otro de los temas abordado en esta memoria de Tesis Doctoral es el de la posible participación de aquaporinas en los cambios inducidos por la simbiosis MA en las plantas hospedadoras en relación con el déficit hídrico. Las aquaporinas son canales de agua que facilitan y regulan el movimiento pasivo del agua a través de un gradiente de potencial hídrico (Luu y Maurel, 2005). Se ha demostrado que la acción concertada de aquaporinas de membrana plasmática y de tonoplasto es la responsable de la osmoregulación del citosol necesaria para que se pueda mantener un metabolismo normal. También se ha comprobado que la acción de las aquaporinas es fundamental para controlar el movimiento del agua en la totalidad de la planta (Grote *et al.*, 1998; Martre *et al.*, 2002; Javot *et al.*, 2003; Aharon *et al.*, 2003). Pero, además, hay estudios que muestran que la expresión de genes que codifican aquaporinas se ve inducida durante la simbiosis MA (Roussel *et al.*, 1997; Krajinski *et al.*, 2000; Marjanovic *et al.*, 2005), lo que sugiere que pueden tener alguna función durante la formación de la simbiosis y/o en la actividad de la misma. De ahí que el estudio de aquaporinas recogido en esta memoria de Tesis Doctoral se iniciara comprobando el efecto que tenia la disminución en la expresión de un gen de aquaporina de tabaco (*NtAQP1*) sobre el patrón de desarrollo de la simbiosis MA y también sobre la eficiencia simbiótica de los hongos MA (evaluada en términos de crecimiento de la planta hospedadora).

Los resultados obtenidos en este estudio demuestran que ambas líneas de plantas utilizadas (de fenotipo salvaje y de fenotipo mutante con un inhibición del 80% en el gen *NtAQP1*) presentaron niveles similares de colonización y de desarrollo de la simbiosis. Sólo se observaron diferencias en base al hongo implicado en la simbiosis. Así la intensidad de colonización y la riqueza de arbusculos generados en estas plantas por *G. mosseae* fue menor que los generados por *G. intraradices*. No obstante, este tipo de resultados se ha observado también en otras plantas y se debe a propiedades intrínsecas de cada hongo (Graham *et al.*, 1996). La falta de efecto de la inhibición del gen *NtAQP1* sobre la capacidad colonizadora de los hongos MA sugiere que bien la función de la proteína NtAQP1 no es relevante para el establecimiento de la simbiosis o bien su inhibición ha sido compensada con alteración de la abundancia de otras aquaporinas (Eckert *et al.*, 1999; Johansson *et al*,. 2000).

La eficiencia simbiótica de los dos hongos utilizados, medida en términos de producción de biomasa por la planta hospedadora, disminuyó en las plantas de fenotipo mutante sometidas a déficit hídrico en comparación con las plantas de fenotipo salvaje. Este efecto puede estar relacionado con el hecho de que esta aquaporina parece funcionar no solo como canal de agua sino también como canal para el CO_2 de manera que tiene importancia fundamental para el mantenimiento de la fotosíntesis en las plantas (Aharon *et al.*, 2003; Uehlein *et al.*, 2003). De hecho, en este estudio también observamos que la disminución del desarrollo de las plantas de tabaco mutantes en condiciones de déficit hídrico fue paralela a la disminución del intercambio de CO_2 de estas mismas plantas. Efecto atribuible, por tanto, a la actividad de la proteína NtAQP1 (Aharon *et al.*, 2003; Uehlein *et al.*, 2003).

El descubrimiento de las aquaporinas en plantas ha significado un cambio importante en la forma de entender las relaciones hídricas de las plantas dado que permite entender que el movimiento de agua a través de las membranas puede ser regulado no solo por fuerzas de origen osmótico sino también mediante modulación de la abundancia y actividad de las aquaporinas (Martre *et al.*, 2002). En cualquier caso, la relación existente entre aquaporinas y las respuestas de las plantas al déficit hídrico es aún un misterio y presenta resultados contradictorios (Aharon *et al.*, 2003; Lian *et al.*, 2004). Pero además, la contribución de las aquaporinas al aumento de tolerancia a la sequía en plantas MA es un hecho que no se ha investigado nunca.

Se ha demostrado que las aquaporinas de membrana plasmática (PIP) resultan reguladas por el déficit hídrico a nivel de trascripción (Mariaux *et al.*, 1998). En el capitulo seis de esta Memoria todos los genes estudiados mostraron su mayor homología de secuencia con PIPs. La hipótesis de partida es que si los hongos MA pueden transferir agua a la raíz de la planta hospedadora, como se ha demostrado en varias investigaciones previas (Hardie, 1985; Faber *et al.*, 1991; Ruiz-Lozano y Azcón, 1995; Marulanda *et al.*, 2003), entonces cabría esperar que la planta deba de aumentar su permeabilidad al agua induciendo la acumulación de aquaporinas a fin de aumentar el flujo transcelular de agua (Javot y Maurel, 2002). Sin embargo, los resultados obtenidos muestran que los genes PIP estudiados resultaban inhibidos tanto en soja como en lechuga por el déficit hídrico y que esa inhibición era incluso más severa en las plantas MA que en las no MA, en contra de nuestra hipótesis inicial. Un resultado similar ha sido obtenido muy recientemente por Ouziad *et al.* (2005) en genes PIP y TIP de plantas de tomate MA sometidas a estrés salino.

En el caso de las plantas de soja el resultado más curioso es que la simbiosis MA adelanta en el tiempo la inhibición originada por la sequía del gen *gmPIP2*. Este hecho puede tener su implicación fisiológica a la hora de ayudar a la planta hospedadora a protegerse del déficit hídrico pues, según Aharon *et al.* (2003), la mayor acumulación de una aquaporina PIP en plantas de tabaco trangenicas puede resultar beneficiosa para la planta en condiciones óptimas dado que permite un mayor flujo de agua. Sin embargo esa mayor acumulación de aquaporinas PIP no tiene efectos beneficiosos bajo condiciones de salinidad o, incluso, resultaba

negativa para la planta, en condiciones de sequía, causando un marchitamiento precoz de las mismas. Por lo tanto, la inhibición de la expresión de *gmPIP2* en plantas de soja MA bajo condiciones de sequía puede constituir un mecanismo de regulación para evitar la perdida de agua de las células de la planta (Barrieu *et al.*, 1999). En apoyo de esta hipótesis, los datos de Ψ en hoja y de CHR muestran que las plantas MA presentaban un mejor estatus hídrico que las plantas no MA.

En el caso de lechuga, los resultados obtenidos con las plantas colonizadas por *G. mosseae* apuntan en la misma dirección, es decir que bajo condiciones de sequía hay una mayor inhibición de los genes PIP en las plantas MA. Sin embargo, las plantas colonizadas por *G. intraradices* no mostraron esa inhibición de los genes PIP a consecuencia del déficit hídrico. Este es un fenómeno con una difícil explicación, aunque hay varios estudios que muestran la existencia de diversidad funcional entre diferentes hongos MA en varios aspectos de la simbiosis. Así, Burleigh *et al.* (2002) estudiaron el efecto de siete especies de hongos MA sobre la expresión de varios genes que codifican transportadores de fosfato en tomate o alfalfa y encontraron que las siete especies afectaban de forma diferente la expresión de cada uno de los genes, como ha ocurrido en este estudio con los genes PIP. En cualquier caso, los resultados obtenidos aquí sobre los genes PIP pueden ser relacionados con los de un estudio previo llevado a cabo también con plantas de lechuga en el que se observó la diferente capacidad de seis hongos MA para captar y transferir agua a la planta hospedadora. *G. intraradices* resultó uno de los hongos más eficientes en esa actividad, mientras que *G. mosseae* mostraba una capacidad mucho menor (Marulanda *et al.*, 2003). Esto sugiere que la estrategia seguida por ambos hongos puede ser también diferente. *G. mosseae*, con menor capacidad de transferir agua a la planta hospedadora podría optar por inhibir ciertas aquaporinas, como hemos observado en este estudio, a fin de conservar el agua existente en la planta. *G. intraradices,* con mayor capacidad para transferir agua a la planta hospedadora podría optar por mantener la permeabilidad radical al agua manteniendo a su vez la acumulación de aquaporinas, como ha ocurrido en este estudio. En cualquier caso, ambas estrategias parecen proteger a la planta de un modo similar pues los resultados de CHR y Ψ en hoja de lechuga eran similares con independencia del hongo que colonizaba la raíz.

En esta memoria de Tesis Doctoral se ha abordado también el estudio de dos genes fúngicos con actividades similares (*Gi14-3-3* y *GiBiP*) dado que codifican proteínas reguladoras y con actividad chaperona que necesitan unirse a otras proteínas efectoras para realizar su función. Las proteínas 14-3-3 son proteínas eucarióticas con un amplio rango de funciones reguladoras de la actividad de proteínas efectoras, funcionando como adaptadores, chaperonas, activadores o represores (Palmgren *et al.*, 1998). De otro lado, las proteínas BiP (del Inglés binding protein) son chaperonas moleculares presentes en el retículo endoplasmático celular. La función de las proteínas BiP es unirse de forma transitoria a otras proteínas que han perdido su estructura terciaria previniendo así su desnaturalización total o precipitación con la consiguiente perdida de su función (Gething y Sambrook, 1992; Hendershot *et al.*, 1996). Tanto las proteínas 14-3-3 como las BiP se han relacionado con la defensa de diferentes organismos

frente a estreses bióticos y abióticos, incluyendo el déficit hídrico (Figueiredo *et al.*, 1997; Alvim *et al.*, 2001; Roberts *et al.*, 2002; Wang *et al.*, 2004).

Los resultados obtenidos demuestran que tanto el gen *Gi14-3-3* como *GiBiP* resultan inducidos por el déficit hídrico (ya sea *in vitro* mediante adición de PEG o *in vivo* sometiendo a las plantas colonizadas por *G. intraradices* a sequía) lo que sugiere que estos genes deben estar implicados en la respuesta del hongo frente a la limitación hídrica. Estos genes pueden estar implicados tanto en la protección del hongo *per se* (inducción de los genes *in vitro*) como en la protección de la planta hospedadora (inducción de los genes durante la formación de una simbiosis MA *in vivo)*.

En el caso de las proteínas 14-3-3 se ha demostrado que uno de sus efectos frente a estreses de tipo osmótico lo llevan a cabo mediante la activación de la protón ATPasa de membrana (Chelysheva *et al.*, 1999; Bavakov *et al.*, 2000; Kerkeb *et al.*, 2002). De hecho, la actividad de las protón ATPasas de membrana está fuertemente regulada por factores que afectan a la fisiología de la célula, como ocurre bajo condiciones de estrés (Palmgren, 1998) y el aumento de la actividad ATPasa es crucial para la actividad de los diferentes sistemas de protección que los organismos vivos han desarrollado frente influencias externas adversas (Serrano, 1989). En el caso de las plantas, la tolerancia a la limitación hídrica depende de los efectos de las proteínas 14-3-3 dado que se ha demostrado que la protón ATPasa de membrana juega un papel fundamental en la regulación del turgor de las células de la planta (Camparot *et al.*, 2003). Así se ha visto que los estreses de tipo osmótico inducen una redistribución de proteínas 14-3-3 entre el citoplasma y la membrana plasmática. Efecto que va acompañado de un aumento notable de la actividad de bombeo de protones de dichas membranas (Chelysheva *et al.*, 1999; Bavakov *et al.*, 2000; Kerkeb *et al.*, 2002).

La función protectora de las proteínas BiP en situaciones de estrés parece estar mediada por una actividad de estas proteínas facilitando la adopción de una estructura terciaria adecuada y la maduración en el retículo endoplasmático de todo el conjunto de proteínas inducido por el déficit hídrico e implicadas en la respuesta al mismo (Ingram y Bartels, 1996; Ricardi *et al.*, 1998; Cascardo *et al.*, 2000; Alvim *et al.*, 2001).

Curiosamente, los resultados de inducción de los genes *Gi14-3-3* y *GiBiP* en las diferentes plantas utilizadas no son uniformes sino que muestran amplia variación en función de la planta hospedadora. En general, las plantas mas sensibles al déficit hídrico, como es el caso de los mutantes antisentido para *NtAQP1* de tabaco (Porcel *et al.*, 2005; Siefritz *et al.*, 2002) presentaron una menor inducción de estos dos genes, lo que parece indicar que, aunque estos dos genes formen parte de los mecanismos a través de los cuales la simbiosis MA aumente la tolerancia de la planta hospedadora frente al déficit hídrico, la contribución de ambos depende de las características fisiológicas de la planta hospedadora.

El conjunto de los resultados obtenidos en esta Memoria de Tesis Doctoral indican que hay, por tanto, unas vías de investigación futura más prometedoras que otras. Así, parece descartable el seguir profundizando en el posible papel de los genes *lea* y *p5cs*. Esto, debe considerarse, no obstante, con cautela dado que no

hemos analizado todos los genes *lea* existentes sino sólo algunos representantes del grupo de las dehidrinas. Sin embargo, los indicios obtenidos en los resultados de estos dos genes (*lea* y *p5cs*) si indican que valdría la pena profundizar en las posibles implicaciones del ABA en la modulación por parte de la simbiosis MA de las respuestas de la planta hospedadora frente al déficit hídrico. Igualmente, los resultados obtenidos con los genes de aquaporinas estudiados sugieren que habría que profundizar en su estudio para correlacionar la inducción o inhibición de los mismos con los niveles de conductividad hidráulica radical y el estatus hídrico de la planta, así como ampliar el número de genes de aquaporina a estudiar. Otro campo de investigación interesante es el que implica a genes que codifican proteínas con actividad antioxidante como la GR, que a nivel bioquímico ha mostrado una inducción importante en plantas micorrizadas. Finalmente, los interesantes resultados obtenidos con genes que codifican proteínas reguladoras y con actividad chaperona como las 14-3-3 y las BiP, cuyo efecto puede ser ejercido sobre un amplio número de proteínas efectoras finales, abre también interesantes perspectivas a la hora de elucidar los mecanismos moleculares implicados en el efecto protector de la simbiosis MA frente al déficit hídrico.

1) La existencia de una actividad GR consistentemente más elevada tanto en raíces como en nódulos de las plantas de soja micorrizadas habría contribuido a disminuir el daño oxidativo a biomoléculas observado en estas plantas y a proteger a las mismas frente al fenómeno de senescencia nodular prematura inducida por sequía.

2) La protección por parte de la simbiosis MA frente al daño oxidativo a biomoléculas y al fenómeno de senescencia nodular prematura inducida por sequía en plantas de soja se produce también mediante mecanismos de evitación primaria del estrés que permiten una mejora del ajuste osmótico y un mantenimiento del potencia hidrico en las plantas MA en condiciones de sequía.

3) La inducción de genes *lea* no parece ser un mecanismo por el que la simbiosis MA proteja a las plantas frente a los efectos negativos del déficit hídrico.

4) La acumulación de genes *p5cs* no parece ser un mecanismo por el que la simbiosis MA proteja a las plantas frente a los efectos negativos del déficit hídrico.

5) La disminución de la expresión de un gen de aquaporina en tabaco no afecta al establecimiento y desarrollo de la simbiosis MA pero disminuye la eficiencia simbiótica de los hongos MA en condiciones de déficit hídrico.

6) Las plantas MA responden ante el déficit hídrico inhibiendo la expresión de los dos genes *PIP* estudiados y anticipando su inhibición en comparación con las plantas no micorrizadas, lo que debe ser considerado como una estrategia dirigida hacia la conservación de agua en la planta.

7) Los genes *14-3-3* y *BiP* de *Glomus intraradices* parecen estar implicados en la protección de las plantas frente al déficit hídrico por parte de la simbiosis MA, indicando que otro de los mecanismos por los que los hongos MA incrementan la tolerancia de la planta hospedadora frente a la sequía puede estar mediado por proteínas reguladoras como las 14-3-3 y por proteínas con actividad chaperona como las BiP, que necesitan unirse a proteínas efectoras para realizar su función, aspectos que hasta ahora nunca habían sido considerados en este campo de la simbiosis MA.

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