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Aislamiento y caracterización de proteínas que interactúan con factores de transcripción MADS box como posibles reguladores implicados en la diferenciación del nódulo fijador de nitrógeno de *Medicago sativa*

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Resumen

El gene *NMH7* codifica a un posible factor de transcripción con dominio MADS expresado en nódulos y flores de *Medicago sativa*. En el presente estudio caracterizamos la expresión de NMH7 en diferentes estadios del desarrollo de *Medicago sativa* así como en el nódulo simbiótico. Los estudios por RT-PCR e inmunodetección mostraron que NMH7 se expresa en semillas y plántulas no inoculadas. Los análisis de plántulas crecidas bajo diferentes condiciones de luz revelaron que la expresión de *NMH7* no es regulada por fitocromo. En semillas, NMH7 se inmunolocalizó en el meristemo embrionario del tallo y de la radícula así como en las células de parénquima. Sin embargo, no se detectó señal en el procambio o en las células del cilindro central de la radícula. En plántulas, el polipéptido de NMH7 se localizó en las células del parénquima cortical, en la región apical y la zona de elongación. En nódulo, la localización de NMH7 coincide con el patrón del ARNm reportado previamente, excepto por la localización de la proteína en la en la primera capa interna de células de córtex del nódulo. Esto se puede deber a movimiento de la proteína entre células a través de plasmodesmos. La presencia de NMH7 en las plántulas no inoculadas sugiere que esta proteína está involucrada en eventos no simbióticos, o bien, particpa en programas relacionados con la colonización bacteriana. Un segundo objetivo del presente trabajo fue la identificación y caracterización de proteínas que interactúan con NMH7. Se empleó una columna de afinidad con un decapéptido derivado del dominio MADS de NMH7 el cual, se ha reportado, media interacciones proteína-proteína con cofactores no MADS. Identificamos dos proteínas con un peso aproximado de ~40 y ~80 kDa que corresponden a la forma monomérica y dimérica de la fructosa-1,6-bisfosfato aldolasa citosólica clase I. Los ensayos de *pulldown* revelaron que las regiones K y C-terminal de NMH7 no participan en la interacción con la aldolasa. Por otra parte, no se requiere la actividad enzimática de la aldolasa para su interacción con NMH7. La aldolasa y NMH7 fueron inmunoprecipitados de extractos de semillas y plántulas no inoculadas. Los estudios de co-localización empleando microscopía confocal, demostraron que tanto la aldolasa como NMH7 se localizan en el citoplasma y en el núcleo de las células corticales. Estos datos sugieren que la aldolasa podría tener un amplio repertorio funcional, como el de otras enzimas glicolíticas.

Por otra parte, usando RT-PCR, Western blotting y microscopía confocal se investigó la respuesta de NHM7 al fósforo (P) y al nitrógeno (N) en etapas tempranas del estableci-

miento de las plántulas de *Medicago sativa*. La respuesta de NMH7 se puede dividir en dos periodos: En el primero, de 1-2 días post-germinación, la expresión del ARNm y la proteína no se afecta por la ausencia o la combinación de ambos macronutrientes en el medio. En el segundo periodo, 3-5 días post-germinación, se observó una regulación negativa en la expresión de NMH7 en las plántulas tratadas con N y P, mientras que las plántulas crecidas en medios sin N y P, NMH7 presenta una expresión constitutiva. Por lo tanto, se propone una acción sinérgica del P y N en la regulación de NMH7. Durante el primer periodo, NMH7 se localiza exclusivamente en el citoplasma mientras que en el segundo, se localiza principalmente en el núcleo. Al igual que en plántulas, el nitrato (NO_3^-) *per se* no inhibe la expresión de NMH7 en nódulos, pero sí afecta su localización celular. Se discuten las implicaciones de estos resultados en la regulación nutricional de NMH7 y su participación en el desarrollo del nódulo.

Abstract

The *NMH7* gene encodes a possible MADS-domain transcription factor expressed in nodules and flowers of *Medicago sativa*. In this study we characterized the NMH7 expression at different stages of *Medicago sativa* development and in nodule. RT-PCR and western blotting analyses revealed that *NMH7* is expressed in non inoculated seeds and seedlings. Examination of seedlings grown under different light conditions revealed that *NMH7* gene expression is not regulated by phytochrome. In seeds, NMH7 was immunolocalized in the shoot and the radicle embryonic meristem, and in parenchyma cells. No signal was detected in the procambium or radicle central cylinder, however. In seedlings the NMH7 protein was specifically localized in the cortex parenchyma of the root tip and in the root elongation zone. In nodules, the localization of NMH7 is coincident with the previously reported mRNA pattern, except for the NMH7 localization in the nodule inner cortex first cell layer. This may be due to protein movement between cells *via* plasmodesmata. The presence of NMH7 previous to bacteria inoculation suggests that this protein is involved in non-symbiotic events or that NMH7 is involved in nodule developmental programs related to bacterial colonization. As second aim of the present work was identify proteins that interact with NMH7. We use an affinity column with a synthetic peptide derived form the MADS domain of NMH7 which has been reported to mediate protein-protein interactions with non-MADS domain interacting protein. We identified ~40 and ~80 kDa specifically bounds proteins as the monomeric and dimeric form of fructose-1,6-bisphosphate aldolase cytosolic class I. NiNTA pull down assay revealed that K- and C-terminal regions of NMH7 are not required for the interaction with aldolase. Aldolase enzymatic activity is not required for the interaction with NMH7. NMH7 and aldolase were coimmunoprecipitated from non inoculated seed and seedlings extracts. Colocalization studies using confocal microscopy showed that aldolase and NMH7 are localized in the cytoplasm and the nucleus of cortical cells. These data together show that *Medicago sativa* aldolase is a novel MADS domain binding protein and suggest a broader functional repertory for this enzyme, as it has been proposed for other glycolytic enzymes.

On the other hand, we investigated the responsiveness of NMH7 to nitrogen (N) and phosphorus (P) in the early stages of development of *Medicago sativa* seedlings using RT-PCR, Western blotting and confocal microscopy. The response of NMH7 can be divided in two periods. The first period is 1-2 days post-germination, during which mRNA and

protein expression are no affected by the presence or absence of these macronutrients in the media in any combination. In the second period (3-5 days post-germination), negative regulation of NMH7 expression was observed in plants treated with N plus P; in plants grown in media lacking both N and P, expression is constitutive. A synergic role of both nutrients in the regulation of NMH7 is proposed. NMH7 is localized in the cytoplasm during the first period, whereas in the second period it is localized mainly in the nucleus. By studying a developmental series of N-starved seed and seedlings, we provided the first evidence for differential sub-cellular localization of NMH7 during seedling establishment. As in seedling, nitrate *per se* is not enough to inhibit NMH7 expression in nodules, but its cellular localization is affected. We discuss the implications of these results in the nutritional regulation of NMH7 and its role in nodule development.

Introducción

El presente trabajo expone los resultados del proyecto de doctorado titulado: «Aislamiento y caracterización de proteínas que interactúan con factores de transcripción MADS box como posibles reguladores implicados en la diferenciación del nódulo fijador de nitrógeno de *Medicago sativa*» realizado en el laboratorio de Ecología Fisiológica del Instituto de Ecología de la Universidad Nacional Autónoma de México. El manuscrito está organizado en cuatro capítulos más tres apéndices. Cada capítulo contiene una breve introducción que delimita el marco conceptual y los avances recientes que engloban los resultados obtenidos expuestos en publicaciones aceptadas o en vías de serlo. La última sección está dedicada a plantear nuevas líneas de experimentación resultado de los trabajos aquí presentados. Los apéndices muestran datos complementarios que por razones de espacio no pudieron ser incluidos en las distintas publicaciones, sin embargo, son parte sustancial del desarrollo metodológico que llevó a la comprensión cabal de los diversos resultados.

Descripción del capítulo I

En este capítulo se documenta la detección de NMH7, tanto el ARNm como proteína correspondiente, en etapas tempranas del desarrollo de *Medicago sativa,* antes del establecimiento de la simbiosis con la rhizobacateria *Sinorizobium meliloti*. Así mismo, se describe cómo se demostró que *NMH7* no es regulado por fitocromo, como ocurre con su ortólogo en *Glycina max* (*GmNMH7*). Todos estos resultados fueron publicados en la revista *Plant Science* (Páez-Valencia et al. 2008 a). Como apéndice mostramos los patrones de expresión de NMH7 en el nódulo de *Medicago sativa:* El ARNm se detectó por hibridación *in situ* y la localización subcelular de la proteína, por inmunofluoriescencia indirecta y microscopía confocal.

Descripción del capítulo II

El capítulo II presenta cómo se llevó a cabo el aislamiento y caracterización de la enzima fructosa-1,6-bisfosfato aldolasa citosólica clase I como una proteína que interactúa con el factor de transcripción NMH7. La interacción inicial se corroboró por ensayos adiciona-

les de *pulldown* e inmunoprecipitaciones recíproca. Los posibles escenarios espaciales de interacción (tejidos, células y compartimentos subcelulares) se establecieron por técnicas de co-localización empleando inmunofluorescencia indirecta y microscopía confocal. Estos resultados se publicaron en la revista *Biochemical and Biophysical Research Communication* (Páez-Valencia et al. 2008 b). Como información complementaria se presenta el apéndice 2 donde se muestran los resultados del análisis de los péptidos (por ES/MS/MS) de las proteínas que interactúan con NMH7. Así mismo, el apéndice 3 muestra de manera detallada la clonación del gene que codifica a la fructosa-1,6-bisfosfato aldolasa citosólica clase I de *Medicago sativa,* el análisis de la secuencia nucleotídica y peptídica y el alineamiento con otras aldolasas citosólicas de leguminosas, monocotiledóneas y gimnospermas.

Descripción del capítulo III

En esta sección se documenta la regulación nutricional por fósforo (P) y nitrógeno (N) de NMH7. Durante este capítulo se señala cómo se demostró que NMH7 es regulado por la acción sinérgica del P y el N durante el establecimiento de la plántula y antes del establecimiento de la simbiosis. Por otra parte, se determinó que en etapas tempranas del desarrollo, NMH7 es localizado exclusivamente en el citoplasma de las células en correlación con la homeostasis de los niveles internos de N; se señala también que después, cuando ocurre un cambio en los niveles endógenos de N, NMH7 es translocado al núcleo indicando qué localización subcelular de NMH7 es regulada por el estado nutricional de la planta y por las condiciones ambientales. También demostramos que en los nódulos tratados con NO_3^-, NMH7 es translocado al citoplasma de las células no infectadas, lo que sugiere un patrón diferencial de localización de NMH7 en condiciones de estrés. Estos datos se exponen en un manuscrito enviado a la revista *Plant Cell and Environment* (Páez-Valencia et al. 2008 c).

Descripción del capítulo de Discusión y conclusiones

En este capítulo se puntualizan las conclusiones generales y, por otra parte, se presentan –a manera de perspectivas– las posibles líneas de investigación que derivarían del presente trabajo.

Capítulo I

Generalidades sobre el establecimiento de la simbiosis entre las rhizobacterias y las leguminosas

La asociación simbiótica entre las raíces de las leguminosas y las rhizobacterias resulta en el desarrollo de un órgano específico llamado nódulo, cuya función principal es la fijación biológica del nitrógeno. Los productos de la fijación biológica del N (aminas en leguminosas temperadas y ureidos en leguminosas tropicales) son exportados desde el nódulo al resto de la planta donde son incorporadas a macromoléculas tales como aminoácidos y proteínas regulando el crecimiento y desarrollo individual y, en el caso de la agricultura, la producción agrícola. El desarrollo del nódulo no solo contribuye de manera crucial a la economía del N de los cultivos de leguminosas, sino que también potencia el contenido de N en el suelo convirtiéndose en componente esencial de la agricultura sostenible. Aparentemente la simbiosis entre leguminosas y rhizobia es benéfica para ambos. Como intercambio por los compuestos nitrogenados exportados, las bacterias simbióticas que pertenecen a los géneros *Rhizobium, Bradyrhizobium, Sinorizobium, Mesorhizobium* y géneros relacionados son abastecidos con energía y esqueletos carbonados, en forma de ácidos dicarbóxilicos a cambio de los compuestos nitrogenados productos de la fijación biológica del N (Lodwig et al. 2003).

El establecimiento de la simbiosis requiere un proceso de reconocimiento y señalización por parte de ambos componentes: planta y bacteria. Bajo condiciones limitantes de N, la planta inicia la síntesis y excreción de flavonoides, los cuales a su vez, regulan la expresión de genes *Nod* de las bacterias de la rizosfera estimulando la síntesis y liberación de moléculas de naturaleza lipoquitoligosacáridas llamadas "factor Nod" (Stouggard 2000). El proceso inicial de infección comienza con la adhesión de la bacteria al pelo radicular, lo que induce su enriscamiento. Las bacterias quedan atrapadas en una oquedad donde la pared celular es degradada localmente, la membrana celular es invaginada y se deposita material vegetal y bacteriano dando inicio a la formación del canal de infección, el cual crecerá hacia la base del pelo radicular y subsecuentemente hacia el córtex (Timmers et al. 1999). Antes de que el canal de infección alcance el córtex, las células corticales se re-diferencían, entran a ciclo celular y son reprogramadas para formar un primordio del nódulo. Cuando el canal de infección alcanza el primordio de nódulo, las bacterias entran a las

células primordiales, a través de un proceso similar a la endocitosis, en el cual, porciones del canal de infección son liberados hacia el interior de las células de la planta. Una vez que están en el citoplasma de las células vegetales, las bacterias –que están rodeadas por una estructura membranosa derivada de la planta llamada membrana peribacteroidal– se diferencían en bacteroides o simbiosomas (Oke & Long 1999). Los bacteroides sintetizan la enzima nitrogenasa que cataliza la reducción del nitrógeno atmosférico (N_2) a amonio (NH_4^+). La sensibilidad de la nitrogenasa al oxígeno contradice las necesidades de ATP del bacteroide para combustionar la fijación biológica del N_2. El abastecimiento continuo de oxígeno y las concomitantes condiciones microaeróbicas se alcanzan mediante la intervención de leghemoglobinas y de una barrera de difusión para el oxígeno en el córtex del nódulo (Ott et al. 2005).

Los nódulos se clasifican como determinados o indeterminados dependiendo de su desarrollo. Los nódulos indeterminados como los presentados por *Medicago sativa, Pisum sativum* y *Vicia hirsuta* son elongados debido a la presencia de un meristemo persistente. En un corte longitudinal de los nódulos indeterminados se distinguen células invadidas en diferentes estadios de diferenciación. La zona I está conformada por pequeñas células meristemáticas que están en constante división y no contienen ningún microsimbionte. La Zona II contiene la zona de infección donde las bacterias son capturadas para la formación de los simbiosomas. En la zona III, las bacterias son albergadas en simbiosomas donde ocurre el proceso de fijación biológica del N. La zona IV no está presente durante las etapas tempranas de diferenciación del nódulo, pero aparece a medida que el nódulo se desarrolla y se hace más prominente conforme la edad. Proximal a la zona senescente está la región V donde las bacterias son esencialmente de vida libre y no presentan características ultraestructurales de simbiosomas (Timmers et al. 2000). Otros tejidos presentes en los nódulos indeterminados son: un córtex externo, una endodermis y un córtex interno llamado parénquima (Van de Wiel 1990). El intercambio metabólico entre los nódulos y los otros órganos de la planta se consigue mediante haces vasculares, localizados en el parénquima del nódulo que se conecta con el sistema vascular de la raíz.

La organogénesis del nódulo –al igual que la de otros órganos tales como flores, hojas y raíces– ocurre a través de muchos pasos de desarrollo regulados espacial y temporalmente. El nódulo es inducido por las rhizobacterias, pero el desarrollo de un nódulo funcional y maduro requiere del intercambio de varias moléculas señales y metabolitos entre la planta y la bacteria. La organogénesis del nódulo es estudiada con la ayuda de bacterias y plantas mutantes, los cuales revelan patrones específicos de diferenciación y con el estudio de marcadores moleculares tanto de procariontes como eucariontes. Por ejemplo, las bacterias mutantes han revelado distintas señales del desarrollo en la progresión de la simbiosis. Las bacterias que no estimulan la división celular necesaria para la formación del nódu-

lo (Nod-) tienen defectos en los genes requeridos para la síntesis del factor Nod (Spaink 2000). Las bacterias que tienen defectos en los polisacáridos de superficie son incapaces de infectar exitosamente a la planta (González et al. 1996). Las bacterias mutantes *bacA* tienen comprometida la integridad de la membrana, senescen tras su deposición en las células vegetales, lo cual indica que se produce un cambio citofisiológico entre el canal de infección y el simbiosoma (Ichige & Walter 1997). Además, las bacterias que sí pueden invadir exitosamente a la planta e iniciar la diferenciación del bacteroide, pero que no pueden fijar nitrógeno, presentan defectos en los componentes enzimáticos o de señalización necesarios para la reducción del nitrógeno molecular (N_2) en amonio (NH_4^+) (David et al. 1988).

Por parte de la planta se pueden identificar cuatro tipos de mutantes simbióticos: mutantes incapaces de formar nódulos (Nod-), mutantes que presentan defectos en la infección, mutantes que pueden formar nódulos pero no fijan nitrógeno (Fix-) y mutantes que presentan un número excesivo de nódulos (Penmetsa et al. 2003). De esta manera, la caracterización de los genes requeridos simbióticamente y su regulación proveen evidencias de los eventos moleculares de la simbiosis. De especial interés son los factores de transcripción debido a que actúan como reguladores maestros potenciales que son capaces de controlar una batería de genes asociados con etapas definidas del programa de desarrollo simbiótico (Yahyaoui 2004). El análisis de los transcriptomas durante el desarrollo y diferenciación del nódulo documentan profundos cambios transcripcionales lo que supone la participación de múltiples factores de transcripción para orquestar los procesos de desarrollo de manera coordinada. Los factores de transcripción conforman 5 % del genoma de las plantas, sin embargo su papel durante el desarrollo y diferenciación del nódulo es un territorio no del todo explorado (Colebatch et al. 2004).

Genes MADS box expresados en el nódulo simbiótico de *Medicago sativa*

Durante la organogénesis floral, cinco tipos de primordios de órganos emergen del meristemo floral y se diferencían en los cuatro órganos florales. A su vez, estos órganos florales están organizados en cuatro verticilos concéntricos: sépalos, pétalos, estambres y carpelos y en el centro de la flor la placenta alberga a los óvulos. La formación específica de los órganos y la ubicación de su desarrollo son determinados por la acción combinada de cinco clases de genes funcionales. Ésto fue dilucidado inicialmente en el modelo ABC, el cual sentó las bases para la comprensión del desarrollo floral y posteriormente se extendió con dos funciones adicionales: la D y E (Coen & Meyerowitz 1991; Pelaz et al. 2001). Casi todos los genes que participan en este modelo pertenecen a la familia de genes MADS box. Una de las características más importantes de las proteínas con dominio MADS es la formación de complejos multiméricos entre sí y, probablemente, también con otras clases

de proteínas (de Folter et al. 2005). Ésto permite crear una gran colección de diferentes complejos transcripcionales que regulan diversos conjuntos de genes blancos, resultando en la formación de órganos específicos en tiempos específicos y posiciones específicas del meristemo floral (Theissen & Seadler 2001). Ente los genes florales MADS box más estudiados están los pertenecientes a la función B, *APETALA 3* (AP3) / *PISTILLATA* (PI) y *DEFICIENS* (DEF) / *GLOBOSA* (GLO) de *Arabidopsis* y *Antirrhinum* respectivamente. Ellos se expresan en el segundo y tercer verticilo, acorde con su función en la especificación de la identidad de pétalos y estambres. Las proteínas AP3 y DEF forman heterodímeros con PI y GLO, respectivamente (Riechmann et al. 1996). Estos heterodímeros son importantes en la regulación de la expresión de *DEF/AP3* y *GLO/PI;* por otra parte, la formación de heterodímeros potencía los niveles de expresión basales y mantienen su expresión (Honma & Goto 2000).

La organogénesis del nódulo ocurre a través de un proceso que se asemeja al desarrollo floral: ambas estructuras emergen a partir de un meristemo indiferenciado. Por lo tanto, es necesario determinar cuál de los genes inducidos en etapas tempranas del desarrollo del nódulo son homólogos de genes homeóticos que intervienen en la identidad de órganos florales. Originalmente el gene *NMH7* se identificó en nódulos de *Medicago sativa,* con base en su alto grado de homología con los genes de identidad de órganos florales de la subfamilia *DEF/AP3.* Su transcrito se localizó exclusivamente en las células infectadas del nódulo y en etapas tempranas del desarrollo floral lo que sugería que el nódulo podría haber reclutado rutas de señalización preexistentes (Heard & Dunn 1995; Zucchero et al. 2001). Posteriormente se identificó un posible ortólogo de *DEFICIENS* llamado *NGL9,* expresado también en nódulos y flores de *Medicago sativa*. Ensayos de retardo y su homología con GLO/PI postularon que NGL9 podría ser un par dimérico de NMH7. Sin embargo, los trabajos no fueron concluyentes (Zucchero et al. 2001).

Estudios de reversión floral en *Glycina max* permitieron la clonación de un posible ortólogo de *NMH7 (GmNMH7)* cuya expresión sugiere una estrecha relación entre su expresión y la inducción floral en soja: (1) los niveles de expresión de *GmNMH7* se incrementan paralelamente al desarrollo floral (tratamientos de días cortos); (2) la señal de *GmNMH7* decrece gradualmente durante el proceso de reversión floral (día corto–día largo) y (3) *GmNMH7* participa en diferentes etapas del desarrollo floral, su expresión se detecta en el meristemo, en el primordio de órganos florales y en órganos completamente diferenciados, lo que sugiere que podría jugar un papel en el mantenimiento de los órganos florales (Wu et al. 2005). La expresión de *GmNMH7* se regula por el fotoperiodo; cuando se induce la floración por días cortos, *GmNMH7* se expresa principalmente en las hojas, en los primordios y en los órganos florales, pero la expresión en los nódulos se reprime; mientras que en días largos, la expresión de *GmNMH7* aumenta en los nódulos y se reprime en las flores

(Wu et al. 2005). Sin embargo, la ausencia de trabajos funcionales, fuera de los organismos modelos bien estudiados, limita el empleo de los patrones de expresión del ARNm para estimar la función de los genes ortólogos y parálogos de la subfamilia *DEF/GLO* (Zahn et al. 2005). Algunos autores proponen una separación entre los bajos niveles de expresión y la función de los miembros de la subfamilia *DEF/GLO* como lo demuestra el hecho de que *DEF* y *AP3* se expresan en sépalos y carpelos, aunque ni la proteína ni los fenotipos mutantes se detectan en estos órganos (Goto & Meyerowitz 1994). Por lo tanto, no se puede asignar una función exclusiva a los genes *DEF/GLO* considerando sólo los niveles de acumulación del ARNm (Zahn et al. 2005; Trobner et al. 1992). Otra característica es la biología celular intrínseca de los factores de transcripción en plantas: en contraste con animales, la continuidad citoplásmica entre células vegetales es la regla y no la excepción. Las células vegetales están conectadas por plasmodesmos, canales alineados de membrana plasmática que proveen una continuidad citoplásmica entre células adyacentes y pueden ser usados para el transporte de ARN, nutrientes y proteínas incluyendo factores de transcripción. Estudios realizados en distintas especies demostraron que los efectos "no autónomos" de los factores de transcripción involucrados en el desarrollo, pueden ser mediados por el movimiento de proteínas entre células (revisado por Wu et al. 2002). Las quimeras periclinales de las mutantes *def* y *glo* generados por alelos que contienen transposones inestables, demostraron que las proteínas DEF y GLO se pueden mover desde las capas internas del meristemo L3 o L2 hacia la L1 para controlar la identidad de los pétalos (Perbal et al. 1996). Sin embargo, AP3 (ortólogo de DEF) en *Arabidopsis* no se mueve entre las células de las capas del meristemo, lo que indica que las diferencias sutiles en la secuencia o diferencias interespecíficas en la maquinaria de translocación afectan el movimiento de factores de transcripción, lo cual es consistente con las diferencias interespecíficas que se han reportado para el movimiento de la proteína verde fluorescente (Crawford & Zambrysky 2001). Estas observaciones enfatizan el cuidado que se debe tener al extrapolar las funciones de un factor de transcripción estudiado en una sola especie o un solo tejido.

En general, los patrones de expresión de los genes MADS se han examinado a nivel de ARNm usando hibridación *in situ*. Sin embargo, como ya se enfatizó, los patrones de expresión del ARNm de ciertos genes MADS y su proteína no siempre se correlacionan. Aunque la expresión de *NMH7* se ha descrito sólo a nivel de ARNm en nódulo y flores, la aproximación empleada para predecir la localización de *NMH7* en nódulos maduros de *Medicago sativa* no ha sido concluyente: se utilizó una sonda derivada de la caja MADS, la cual representa la región más conservada de estos genes y no es resolutiva para discernir patrones específicos.

El propósito de la primera parte de la tesis fue identificar y localizar la proteína NMH7 en diferentes etapas del desarrollo vegetativo de *Medicago sativa,* incluyendo semillas, plántulas no inoculadas y en nódulos, empleando anticuerpos policlonales considerando que la naturaleza tetraploide de *Medicago sativa* no permite un análisis genético convencional como los aplicados en otros sistemas de fácil transformación. El análisis de la expresión de NMH7 en etapas tempranas se justifica por ciertos estudios funcionales en los cuales se ha reportado que algunos de los genes MADS box que se expresan en etapas tempranas del desarrollo de la semilla de *Medicago truncatula* también se detectan en flores (Verdier et al. 2008). Además, si NMH7 interviene en el programa de desarrollo del nódulo, éste podría ejercer su función antes del establecimiento de la simbiosis y ser regulado por estímulos ambientales como la luz, de la misma manera que *GmNMH7.* Por lo tanto, el objetivo del presente trabajo fue (1) analizar si el ARNm de *NMH7* y su correspondiente polipéptido están presentes antes de la inducción del nódulo, (2) determinar si la luz como señal ambiental regula la expresión de *NMH7* en *Medicago sativa,* (3) corroborar por hibirdación *in situ* el patrón de expresión de *NMH7* empleando como sonda la región codificante de la caja K-C y (4) correlacionar el patrón de expresión del ARNm con la localización subcelular de NMH7 en los distintos tipos celulares del nódulo maduro de *Medicago sativa*.

Resultados

Artículo:

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Localization of the MADS domain transcriptional factor NMH7 during seed, seedling and nodule development of *Medicago sativa*

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ABSTRACT

The *NMH7* gene encodes a possible MADS-domain transcription factor expressed in the nodules and flowers of *Medicago sativa*. In this study we characterized NMH7 expression at different stages of *M. sativa* development and in nodules. RT-PCR and western blot analyses revealed that *NMH7* is expressed in seeds, cotyledon seedlings, and primary roots. Examination of seedlings grown under different light conditions revealed that *NMH7* gene expression is not regulated by phytochrome. In seeds, NMH7 was immunolocalized in the shoot and radicle embryonic meristems, and in parenchyma cells. No signal was detected in the procambium or radicle central cylinder, however. In seedlings, the NMH7 protein was specifically localized in the cortex parenchyma of the root tip and in the root elongation zone. The localization of NMH7 is coincident with the previously reported mRNA pattern, except for the localization of NMH7 in the nodule inner cortex first cell layer. This may be due to protein movement between cells via plasmodesmata. The presence of NMH7 prior to bacteria inoculation suggests that this protein is involved in non-symbiotic events or that NMH7 is involved in nodule developmental programs related to bacteria colonization.

1. Introduction

MADS domain proteins are transcription factors fundamental to the developmental control of the signal transduction process in eukaryotes [1]. MADS-box genes contain a highly conserved sequence of 180 bp, named the MADS-box, which encodes a DNA-binding domain [2]. MADS proteins can be classified as type I or type II [3]. Type II proteins contain four domains, MADS, I (intervening), K (keratin-like), and C (C-terminal), referred to as MIKC-type proteins. Molecular and genetic studies in *Antirrhinum majus*, *Arabidopsis thaliana* and *Petunia hybrida*, have shown that MADS-domain proteins play an important role in plant development. Although most information refers to the function of MADS-domain proteins in the differentiation of floral organs, recent evidence indicates the involvement of MADS proteins in vegetative development including embryo, root, leaves, and nodules [4]. In general, the expression pattern of plant MADS-box genes has been examined mainly at the mRNA level using *in situ* hybridization. The MADS-box transcription factors, DEFICIENS and GLOBOSA, are capable of trafficking between cells via plasmodesmata from the inner cellular layers L2 or L3 to L1 to control petal identity acting non-cell autonomously in developing flowers [5]. Also, non MADS-box transcription factors like KNOTTED 1 (KN1) have a similar behavior; *KN1* mRNA is expressed in the inner cellular layers of the maize shoot apical meristem, whereas KN1 protein is localized in nuclei of all layers, indicating protein movement from L2 and L3 to the L1 protodermal layers [6]. In addition, environmental factors such as temperature and day-length have profound effects on the expression and activity of MADS-box genes, such as those found in tomato plants [7]. Therefore, an integral study of MADS-box plant transcription factors should consider the correlation between mRNA and protein and the factors that directly or indirectly regulate their expression. The *NMH7* gene, expressed in *Medicago sativa* root nodules and flowers, is a possible ortholog of the Antirrhinum *DEFICIENS* (*DEF*, 54% identity), a B-function gene involved with *GLOBOSA* (*GLO*) in the development of flower sepals and stamens [8]. It has been proposed that *NMH7* could represent MADS-box gene recruitment from the cluster of B-function genes

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to perform a specialized function in legume plants [9]. Although there is published information about *NMH7* expression at the mRNA level in flowers and nodules of *M. sativa*, no reports exist about NMH7 localization. Recently there have been reports that the possible ortholog (GmNMH7) mRNA of *Glycina max* is regulated by photoperiod expression in nodules and flowers [10]. The purpose of the present study was to identify whether the *NMH7* transcript and the corresponding polypeptide are present prior to the induction of nodule development, and to determine whether light, as an environmental signal, is able to regulate the *NMH7* expression in *M. sativa*.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *M. sativa* were surface-sterilized with 2.5% sodium hypochlorite and rinsed five times with distillated water. The seeds were soaked for 2 h in distilled water and were then transferred to Petri dishes covered with moistened filter paper and germinated at 20 °C in the dark for 48 h. For the inoculation assay, 1-week-old seedlings were grown on vermiculite in controlled greenhouse conditions and were inoculated with a *Sinorhizobium meliloti* strain, 1021 (2×10^5 cells). The inoculated plants were subsequently supplemented with Fahreus medium without nitrogen.

2.2. Light treatment

Three groups of 50 seeds were spread on a layer of paper filter moistened with water. For each treatment, three replications of 50 seeds were spread on a layer of paper filter moistened with water and were incubated 2 h in darkness. Subsequently, they were irradiated with R (red light), FR (far red light), R + FR or R + FR + R for 15 min each; at the end of each of the four treatments, seeds were returned to the dark for 48 h at 20 °C. Reproducibility of the data was confirmed in three independent experiments. For the R treatment, the Petri dishes were placed inside the boxes made from red plexiglass filters (series No, 2424 Röhm & Hass, México, D.F., R:FR = 4.1, FFD = 1.3 μmol m^{-2} s^{-1}). For the FR treatment, the Petri dishes were placed inside boxes made from red and blue plexiglass filters (series No. 2423, R:FR = 0.30, FFD = 5.19 μmol m^{-2} s^{-1}). All treatments were carried out in a dark room with a green safe light, as well as incandescent and fluorescent lights, which were illuminated when appropriate. The R, FR and R:FR ratio were measured with a SKR-100 radiometer (Skye Instruments, Skye, UK).

2.3. RT-PCR analysis of gene expression

RT-PCR was performed according to Halford et al. [11]. Total RNA from young seedlings treated under different light conditions was purified with TRIZOL reagent (Invitrogene) according to the manufacturer's conditions. For dry seeds, total RNA was isolated following the procedure of Li and Trick [12]. RNA was quantified spectrophotometrically, separated by formaldehyde gels, and stained with ethidium bromide to ensure RNA integrity and equal loading of samples. cDNA was synthesized from 1 μg of total RNA using the Super Script II RNase H Reverse transcriptase system (Invitrogene). Msc27 constitutive expression was used as an RT-PCR control [13]. Primer sequences for PCR were as follows: for NMH7 complete coding sequence (Accesion number Gene Bank L41727.1) NMH7F 5′CCATGGCTCGAGGAAAGATC3′ and NMH7 R 5′GGATCCGGTGAGATCCGAG3′; for Msc27 the Msc27F 5′GGAGGTTGAGGGAAAGTGG3′ and Msc27 R 5′CACCAACAAAGCAAAGAATTGAAGG3′. The PCR products were extracted and ligated into the poly-T site of pGEMT Easy vector (Promega USA), and the complete sequence was determined.

2.4. Expression and purification of the K-C domain of NMH7

The coding sequence of the K-C domain of NMH7 was amplified by PCR using template nodules of cDNA. The two primers used were: NMH7KF 5′*CATGG*AAGATGTCAATAG3′ and NMHCR 5′*GGATCC*GGGTGAGATCCGAG3′ adding an NcoI restriction site at the 5′ end and a BamHI at the 3′ end, respectively (underlined in the sequence). The 410 base pair product was cloned into the pGEMTeasy vector and was sequenced. A positive clone was digested with the NcoI and BamHI, and the fragment released was purified using the QUIAquick gel extraction kit (QUIAGEN). The fragment was subcloned into the NcoI–BamHI restriction site of the pQE60 (QUIAGEN USA), and the plasmid was transfered to the *Escherichia coli* strain M15. A positive clone was first cultured overnight in 6 mL of Luria-Bertani (LB) medium (25 μg mL^{-1} kanamycin and 100 μg mL^{-1} ampicilin) at 37 °C. A 5 mL aliquot of the overnight culture was transferred to 100 mL of LB medium and was allowed to grow until the A_{600} reached 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and was allowed to induce for 5 h. Cells were harvested by centrifugation, and the purification of the recombinant protein was done under denaturing conditions by Ni-NTA affinity chromatography according to manufacturer's instructions.

2.5. Antibody production

For polyclonal antibody production, one white New Zealand female rabbit was immunized through multiple subscapular injections with the K–C recombinant domain of NMH7 (100–250 μg) emulsified with adjuvant. Freund's complete adjuvant (Sigma USA) was used for the first inoculation and incomplete adjuvant for the subsequent immunization at 3-week intervals. The titer and specificity of the antibodies was monitored through inoculation protocol. Pre-immune and immune sera were collected and used without any subsequent purification process. Cross-reactivity was tested in the protein crude extracts of seed cotyledons and in the primary roots of seedlings.

2.6. Western blot analysis

Frozen plant tissue was ground in extraction buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM PMSF, 0.5 μL/mL leupeptine). The crude extract was centrifuged at 15,000 × *g* for 30 min, and the supernatant was recovered. The protein concentration was determined by a dye-binding assay (Bradford Coomassie reagent, Amresco). For western blotting, the protein samples (15 μg) were separated by SDS-PAGE according to the method of Laemmli [14] and were transferred onto Immobilon-P membranes (Milipore USA) with a Hoefer transfer tank unit at 180 mA for 1 h at 4 °C. The filters were blocked in 5% dry milk in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.075% Tween-20) for 1 h and were washed three times with TBST. Polyclonal antibodies, anti-NMH7 K-C domain, were used at 1:2000 dilution in TBST with 5% (w/v) dry milk. After three washes in TBST, the membranes were incubated for 1 h with 1:2500 dilution of donkey anti-rabbit IgG horseradish peroxidase-linked whole antibody (Amersham). Each membrane was again washed three times in TBST, and the detection of antigen–antibody complex was performed using the Immobilon Western Chemiluminescent HRP substrate (Milipore, USA).

2.7. Tissue embedding and sectioning

Seedling and nodules (30 dpi) were fixed in FAA (3.7% Formaldehyde, 50% Ethanol, 5% Acetic Acid) for 3 h at room temperature. Fixed material was dehydrated in a graded ethanol series (50%, 60%, 70%, 80%, 90%, 96% and 100%), and the ethanol absolute was then replaced by a histological clearing agent (Histochoice clearing agent 1× agent Amresco). The tissue was embedded in paraplast at 60 °C. The embedded tissue was sliced into 10 μm sections using a microtome (Leica instruments) and was placed onto Fisher Biotech "probe-on" microscope slides (Fisher Scientific). The seeds were fixed in FAA for 3 h at room temperature, dehydrated in an ethanol series (25%, 50%, 75% and 100%), and then infiltrated with t-butyl alcohol (Sigma, USA) diluted in ethanol in a stepwise series (5%, 50%, 75% and 100%) at 60 °C, followed by infiltration with paraplast and several changes of pure molten paraplast at 60 °C. After paraplast infiltration, the samples were placed in blocks and allowed to harden. The slide sections, 5–10 μm, were placed on to Fisher Biotech "probe-on" microscope slides (Fisher Scientific).

2.8. NMH7 immunolocalization

Slides with tissue sectioning were deparaffinized with xylene and were rehydrated through a graded ethanol series. After deparaffination and hydratation, the slides were washed in PBS. To recover antigenicity histological sections, they were immersed in antigen retrieval citra plus solution (BioGenex HK080-5K) and autoclaved for 5 min. The slides were pretreated with a power universal reagent (BioGenex HK085-5K) to reduce non-specific binding and then were incubated with the primary anti NMH7 K-C domain serum, at a 1:200 dilution in PBS as well as pre-immune rabbit serum (negative control, supplementary material). Three rinses with PBS were followed by incubation with a horseradish peroxidase polymeric rabbit/mouse system (DAKO Corp K067589) at a 1:200 dilution in PBS. After several washes, the immunohistochemistry preparations were contrasted with hematoxylin.

3. Results

3.1. Specificity of a polyclonal antibody against the K-C domain of NMH7

The complete coding sequence of K-C domain of NMH7 was cloned in to a pQE60 expression vector and was transformed into *Escherichia coli*, and the over-expressed protein was purified on a Ni-NTA affinity column under denaturing conditions. The purified polypeptide was used for immunization of a female rabbit. The immunoblot analysis of NMH7 reveled that sera of a rabbit immunized with the K-C recombinant domain of NMH7 recognizes only a 29 kDa polypeptide within an extract from IPTG-induced cells of *Escherichia coli* transformed with the complete coding sequence of NMH7 (MIKC) (Fig. 1, AI). As a negative control, we used the crude extract of non-induced cells, and no signal was detected (Fig. 1AII). The polyclonal sera specifically recognizes the 29 kDa polypeptide, which is the translation product of NMH7 mRNA, in protein crude extract of *M. sativa* seedlings, and coincides with the predicted size for this protein (Fig. 1BIII).

3.2. Expression of NMH7 in seed and seedlings of M. sativa

Expression of *NMH7* in seeds and young seedlings was analyzed by RT-PCR using specific primers for the coding region of *NMH7*. mRNA templates were prepared from dry seeds, roots, and cotyledons from young seedlings. Complementary cDNA synthesized from mRNA was used in RT-PCR. The presence of the NMH7 protein in different tissues was analyzed by immuno-

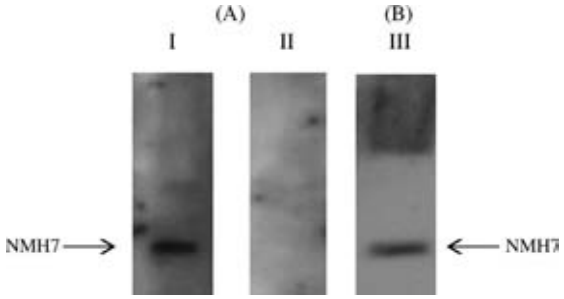

Fig. 1. Immunoblot analysis of NMH7. (A) Immunoblot analysis of induced (3 h) crude extract of recombinant bacteria: the sera from a rabbit inoculated with the K-C domain of NMH7 recognized the complete recombinant protein (MIKC) expressed in *Escherichia coli* strain M15 (I). As negative control, the polyclonal sera anti K-C NMH7 domain does not cross-react with any protein of the non-induced bacterial crude extract (II). (B) Specific identification of a 29 kDa polypeptides in protein crude extracts of *Medicago sativa* seedling (III).

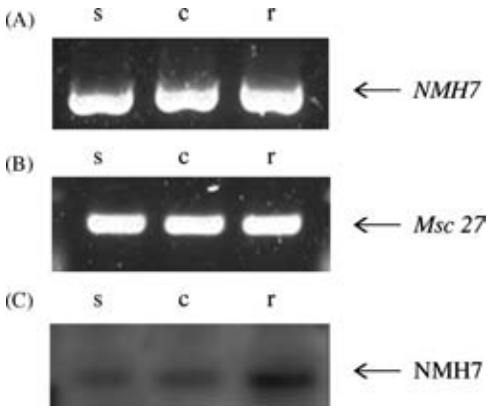

Fig. 2. Analysis of NMH7 expression during early stages of *M. sativa* development: dry seeds (s), cotyledon (c), and primary root (r) of seedling. (A) RT-PCR analysis of the expression of *NMH7* from *M. sativa* in various tissues. (B) *Msc 27* primers were included in each reaction as a loading control. (C) Detection of NMH7 protein in seeds and seedling tissues by immunoblott analysis. Membranes were incubated with an antibody raised to recombinant K-C domain of NMH7, which reacts with a 29 kDa in crude extracts from s, c and r. Reproducibility of data was confirmed in three different experiments.

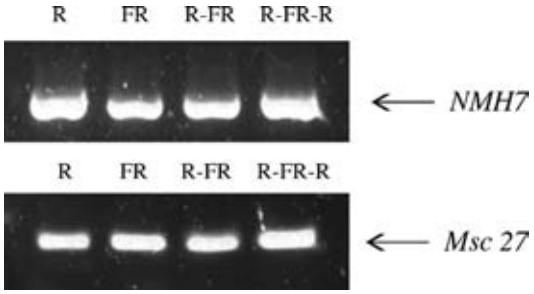

Fig. 3. Expression of *NMH7* under different light conditions. *M. sativa* imbibed seeds were spread on a layer of filter paper moistened in water. Then, seeds were treated with R (red light), FR (far red light), R–FR, or R–FR–R for 15 min each and then were returned to the dark for 48 h. Seedlings were harvested, and RT-PCR was used to analyze the expression of *NMH7*. *Msc 27* primers were included in each reaction as a control. Reproducibility of data was confirmed in three different experiments. There were no differences in the expression levels of *NMH7* in the different light treatments.

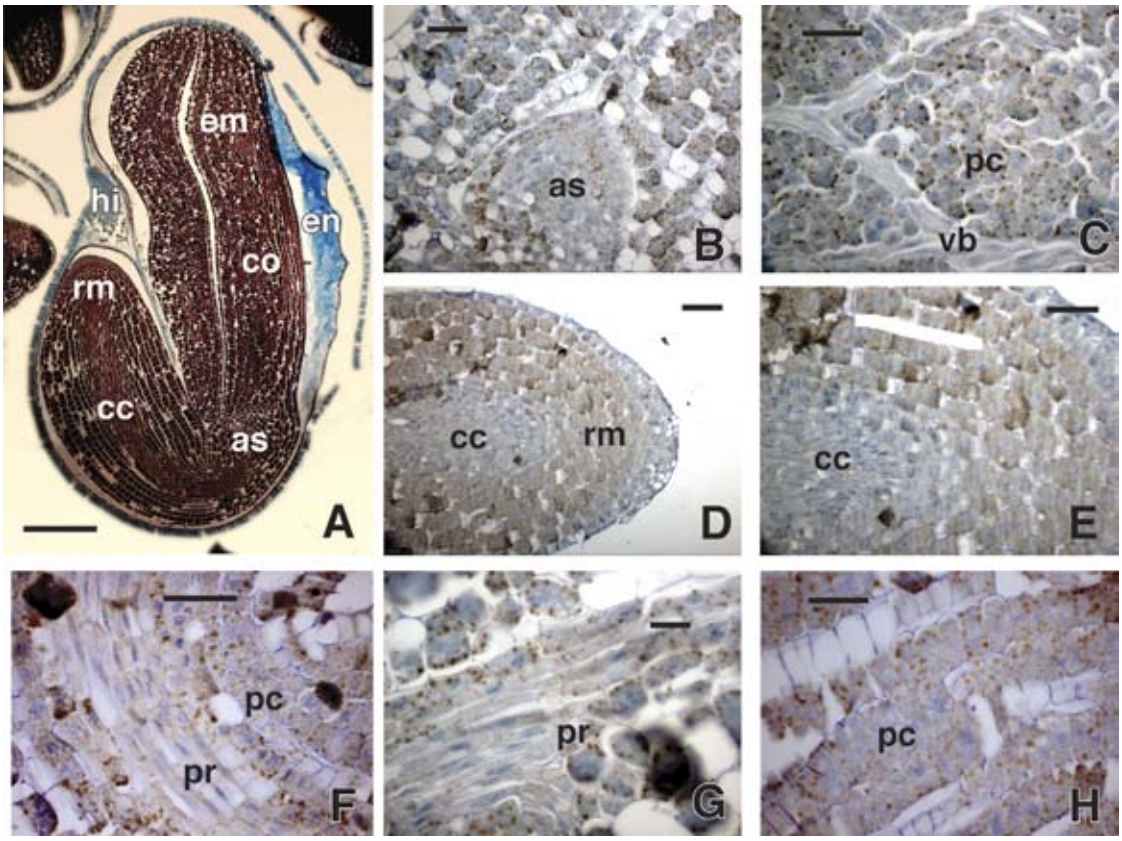

Fig. 4. Immunolocalization of NMH7 in mature seeds of *M. sativa*. (A) Section of a *M. sativa* mature seed contrasted with Mason's staining. (B) Sections probed with an anti-sera raised against K-C NMH7 recombinant domain results in localization signals in the embryonic shoot apical meristem and cotyledon cells. (C) NMH7 localization in cotyledonary tissues shows a punctuate pattern. Non-signal was detected in the vascular bundles. (D) Localization of NMH7 in radicle meristem. (E) Higher magnification of the radicle meristem cells where NMH7 localization signal contrasts with the non-signal (blue) of the central cylinder. (F) Differential signal pattern between procambium and parenchyma cells. (G) Higher magnification of the procambium cells showing a slight and diffuse signal and of the parenchyma cells with a punctuate signal. (H) The remaining parenchyma cells of the radicle also shows a punctuated pattern. Abbreviations: (as) apical shoot meristem, (co) embryonic cotyledon, (em) embryo, (en) endosperm, (hi) hilum, (pc) parenchymal storage cells, (pr) procambium cells, (cc) central cylinder, (rm) root meristem (vb) vascular bundles. Bars A = 0.2 mm, B,C, F, and H = 20 μm, E and G = 20 μm, D = 30 μm.

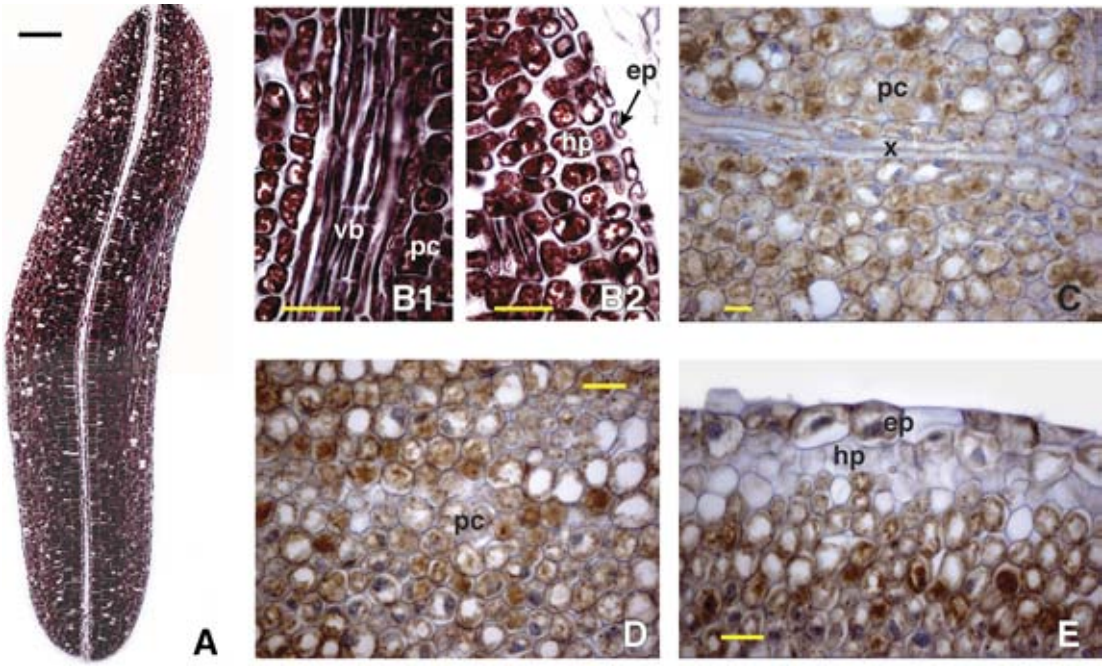

Fig. 5. Immunolocalization of NMH7 in cotyledon of non-inoculated seedlings. (A and B) Sections of *M. sativa* cotyledons stained with Mason's staining. (C) NMH7 localization in seedlings parenchyma cells, no signal is detected in xylem. (D) The remaining parenchyma cells show a diffuse pattern of NMH7 localization. (E) NMH7 localization in hypodermal and epidermal cells. *Abbreviations*: ep (epidermis), hp (hypodermal cells), pc (parenchymal storage cells), x (xylem). Bars A = .2 mm, B = 50 μm, D and E = 40 μm.

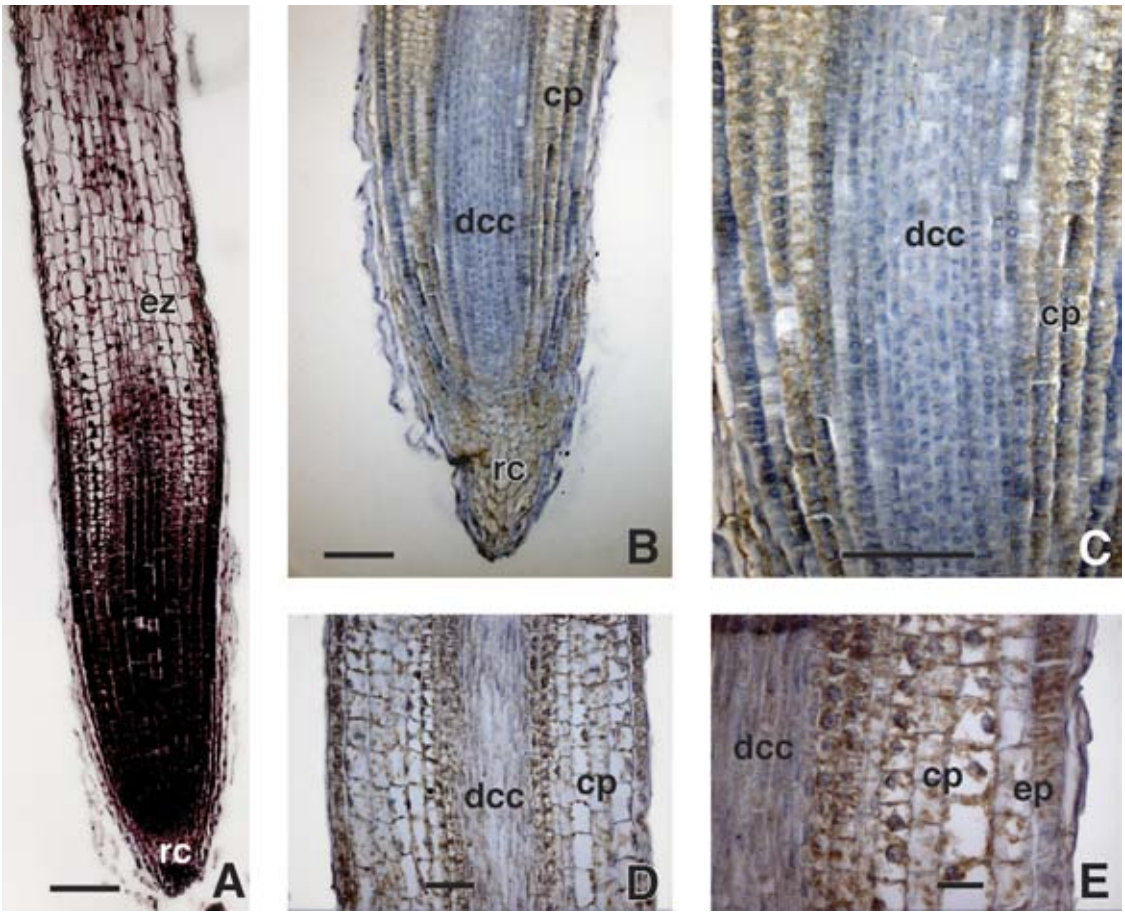

Fig. 6. Immunolocalization of NMH7 in the primary root of *M. sativa*. (A) Medial longitudinal sections of *M. sativa* root cap and elongation zone contrasted with Masson's staining. (B) NMH7 is immunodetected in the root cap and in the expanding cortex parenchyma. No signal was detected in the developing central cylinder. (C) Higher magnification of the developing central cylinder and expanding cortex parenchyma. (D) Immunolocalization of NMH7 in elongation zone. NMH7 protein is detected in the epidermis and the cortex parenchyma. No signal was detected in the developing central cylinder. (E) Higher magnification of the cortex parenchyma corresponding to the elongation zone. *Abbreviations*: root cap (rc), developing central cylinder (dcc), cortex parenchyma (cp), elongation zone (ez), epidermis (ep). Bars A = .3 mm, B = 150 μm, C = 100 μm, D and E = 20 μm.

blotting to determine the co-translation of the mRNA detected in the same tissues. Total soluble proteins were extracted from seed, cotyledons and primary roots of *Medicago* seedlings and then were immunoprobed with the polyclonal antibodies against the K-C domain of NMH7. Both mRNA and protein were detected in seeds and in seedlings (Fig. 2A and B), indicating that NMH7 (mRNA and protein) is present before bacterial colonization. These results modify the previous working hypothesis where the presence of NMH7 results in the activation of specific target genes whose product directly or indirectly defines the differentiated state of infected cells, since NMH7 is present before the bacterial colonization.

3.3. The expression of NMH7 at early stages of M. sativa development is not regulated by phytochrome

The expression of *NMH7* in seedlings treated with different light conditions was analyzed by RT-PCR. The mRNA accumulation of *NMH7* and *Msc 27*, used as a constitutive control, were analyzed. No significant differences in the *NMH7* levels were observed in the different light treatments; therefore, the *NMH7* expression is not photoreversible (Fig. 3). These results show that in *M. sativa* seedlings, the transcript *NMH7* levels are not regulated by phytochrome.

3.4. Immunolocalization of NMH7 in early stages of M. sativa development

Longitudinal sections of mature seeds and 2-day-old seedlings were immunoprobed with the polyclonal antibody raised against the K-C recombinant domain of the NMH7 MADS-domain transcription factor and were revealed with the horseradish peroxide polymeric rabbit/mouse conjugated system. The signal was detected as a brown precipitate contrasting against the blue hematoxylin staining that reveals the cellular sites where the protein is not detected. In seeds, NMH7 is expressed with a punctuate pattern mainly in embryonic tissues such as the embryonic shoot and root apical meristem, specifically in parenchymal cells either from cotyledons or embrionary roots (Fig. 4B–E and H). NMH7 was slightly detected in the cotyledon procambium and in the central cylinder of embryonic radicles showing a diffuse pattern (Fig. 4F and G). NMH7 was also not detected in endosperm, hilum, or seed coat tissues (data not shown).

In the seedlings cotyledons, NMH7 protein was detected in the epidermal, hypodermal, and storage parenchymal cell but never in xylem tissues (Fig. 5C–E). The signal had a diffuse pattern in seedlings in contrast to the seed. In longitudinal sections of primary root seedlings, NMH7 protein was detected in the root tip

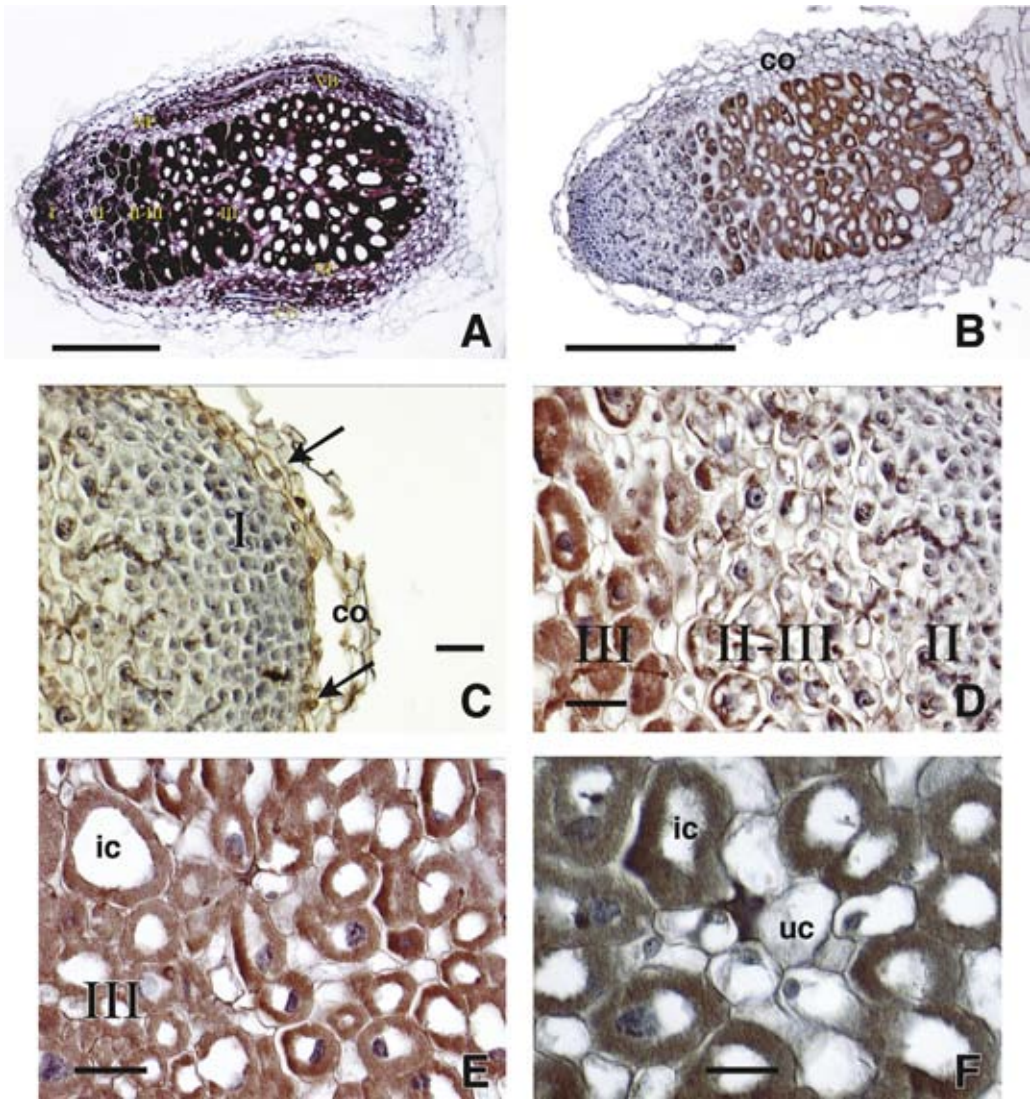

Fig. 7. Immunolocalization of NMH7 in *M. sativa* nodules. (A) Longitudinal sections of *M. sativa* nodules stained with three chromic Masson's staining. longitudinal sections through *M. sativa* nodules. (B) Sections incubated with the anti-K-C NMH7 domain antibody resulted in the localization signals in the symbiotic zone, no signal was detected in peripheral tissue such as the cortex, endodermis and vascular bundles. (C) In the meristem zone just the first layer present visible signal (arrows). (D) Note an increase in the signal from zone II to zone III. (E) The NMH7 protein was detected in the infected cells. (F) Non-signal was detected in uninfected cells and appear blue due to the absence of signal. *Abbreviations*: nodule parenchyma (NP), nodule cortex (co), nodule endodermis (NE), vascular bundles (VB) uninfected cells (UC), infected cells (IC). Bars A = .25 mm, B and E 25 μm, C and D 20 μm.

region, preferentially in the root cap and in cortex parenchyma of the root tip, but no signal was detected in the developing central cylinder (Fig. 6B and C). In the elongation zone, NMH7 was immunolocalized in epidermal cells, cortex, and endodermis but no signal was detected in the vascular cylinder (Fig. 6D and E). In both structures, there is a predominantly cytoplasmatic protein detection that is contrasted by the blue hematoxylin staining in the nucleus, but the resolution of this technique is not reliable for accurate description of protein subcellular localization.

3.5. Immunolocalization of NMH7 in nodules

We performed immunohistochemistry to localize NMH7 within mature nodules. For the structural description of nodules, the nomenclature of Vasse et al. [15] was followed: Meristem (zone I), invasion zone (zone II), interzone (II–III), N_2-fixing or infected zone. NMH7 was detected in the inner layer of the cortex (Fig. 7C), zone II, interzone II–III and zone III (Fig. 7D). No signal was detected in uninfected cells (Fig. 7F), meristem, endodermis, or vascular tissues (Fig. 7B). These results are coincident with the mRNA expression pattern previously reported [8], except for localization of the NMH7 protein in the inner layer of the nodule cortex close to the meristamic region.

4. Discussion

For a number of MADS-box genes, the only available information is the expression pattern at the mRNA level. Although there is a general correlation between the expression pattern and place function, the presence of a transcript in a given tissue does not necessarily imply that gene activity is necessary for its proper development. The expression pattern of MADS-box RNA and the corresponding protein might not correlate; for example, DEFICIENS and GLOBOSA act autonomously in developing flowers [5]. For this reason, we here perform an integrated study of NMH7 expression

at early stages of *M. sativa* development and nodule using RT-PCR, western blotting, and immunolocalization approaches.

As nodulation evolves after the separation of flowering, it is possible that the genes required for nodule development were recruited from reproductive pathways [16]. A recent example of this phenomenon has been observed in specific cystein cluster proteins (CCPs) which have a potential role in the defense against pathogens, but might also act as signaling molecules that regulate the development of seeds and nodules [17]. These recruitment phenomena could explain the expression of NMH7 in different plant tissues. In the present work, both the NMH7 protein and the mRNA were detected in seed and seedlings. In seeds, NMH7 protein was detected in the epidermal, hypodermal, and parenchymal cells; NMH7 could be involved in a non-symbiotic mechanism related to seed and seedling development.

Previous studies have shown that *Medicago* seeds and seedlings that have been treated with Nod factor and inoculated with a limiting population of *S. meliloti* have an increase in the number of nodules per plant, showing that seeds are receptive to submicromolar concentrations of Nod factor, suggesting the possibility of a high affinity Nod factor perception system in seeds or embryos [18]. NMH7 expression in seeds and seedlings could be a part of this regulatory network that "memorizes" the contact with the Nod factor, improving the activation of the developmental program.

Transcription factors are regulated by developmental cues and environmental factors so as to orchestrate plant development and organogenesis. The soybean gene *GmNMH7* (*Glycine max NMH7*-like gene) involved in flower reversion is regulated by the photoperiod in nodule and flowers [10]. Our data suggest that the expression of *NMH7*, in a similar way to germination, is not dependent of R or FR, suggesting in turn that phytochrome does not mediate *NMH7* transcript accumulation in *Medicago* seedlings. However, light responsiveness may be a target of artificial selection in domesticated plants [19]. In seedling primary roots, the root cap and the epidermis appear to play fundamental roles in sensing external stimuli, affecting root growth and morphogenesis. For example, the legume plants growth under nitrogen limitation, express high levels of chalcone synthase at the root tip, and the main population of nod-inducing flavonoids is excreted from the root elongation zone, beginning a signalling dialogue between the host plant and the bacteria initiating nodule formation [20]. The involvement of MADS-box genes in flavonoid biosynthesis has recently been demonstrated. Synthesis of anthocyanins from sweet potato is regulated by the MADS-box gene, *Ib MADS 10*, while DEFICIENS, the putative orthologe of NMH7 in *Antirrhinum,* regulates the flavonoid biosynthesis responsible for petal pigmentation involved in pollinator preferences [21-23]. NMH7 protein expression was detected in the cortex parenchyma of the root tip and in the epidermis, and cortex of the elongation zone. Therefore, NMH7 could be able to regulate the expression of proteins involved in the biosynthesis of the flavonoids that regulate the synthesis of Nod factors and trigger nodule morphogenesis.

No cell autonomous effect has been described for several plant transcription factors by immunodetection as a consequence of a protein movement between cells via plasmodesmata [24]. A significant increase in nodule number was promoted by a viral movement particle transgenic expression in *Medicago truncatula*, which increased plasmodesmata permeability, leading to cell to cell movement in this legume. This developmental nodule response demonstrates a primary role for plasmodesmata-mediated trafficking in nodule development [25]. In *M. sativa* nodules, NMH7 protein was detected in infected cells (zone III), the invasion zone (zone II), and the interzone (II–III). These results partially correlate with the MADS-box genes expression in nodules as determined by *in situ* hybridization using the conserved *NMH7* MADS-box region. NMH7 protein was also present in the inner layer of the nodule cortex, although no gene transcript was detected. NMH7 can move through nodule plasmodesmata, acting as a sensor of environmental signals integrating the physiological stimuli with the developmental program, as described for other MADS-box genes.

NMH7 may participate in regulatory networks involved in seeds, seedling, and nodule development; however, its specific role in the biology of *M. sativa* will be determined by additional biochemical and genetic functional studies.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2008.06.008.

References

[1] F. Messenguy, E. Dubois, Role of MADS box proteins en their cofactors in combinatorial control of gene expression and cell development, Gene 316 (2003) 1–21.
[2] S. De Bodt, J. Raes, Y.V. Van de Peer, G. Theissen, And then there were many: MADS goes genomic, Trends Plant Sci. 8 (2003) 475–483.
[3] E. Alvarez-Buylla, S.J. Liljegren, S. Pelaz, S.E. Gold, C. Burgeff, G.S. Ditta, L.R. De Pouplana, L. Martínez-Casillas, M.F. Yanofsky, An ancestral MADS box gene duplication occurred before the divergent of plant and animals, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 5328–5333.
[4] F. García-Maroto, M.J. Carmona, J.A. Garrido, M. Vilches Ferrón, J. Rodríguez-Ruiz, D. López Alonso, New roles for MADS-box genes in higher plants, Biol. Plant. 43 (2003) 321–330.
[5] M.C. Perbal, G. Haughn, H. Saedler, Z. Schwarz-Sommer, Non cell autonomous function of the Antirrhinum floral homeotic proteins DEFICIENS and GLOBOSA is exerted by their polar cell-to-cell trafficking, Development 122 (1996) 3433–3441.
[6] W.J. Lucas, S. Bouche-Pillon, D.P. Jackson, L. Nguyen, L. Baker, B.S. Ding, S. Hake, Selective trafficking of KNOTTED 1 homeodomain protein and its mRNA through plasmodesmata, Science 270 (1995) 1980–1983.
[7] S.R. Hepworth, F. Vlaverde, D. Ravenscroft, A. Mouradov, G. Coupland, Antagonic regulation of flowering-time gene *SOC1* by *CONSTANTS* and *FLC* via separate promoter motifs, EMBO J. 21 (2002) 4327–4337.
[8] J. Heard, K. Dunn, Symbiotic induction of a MADS-box gene during development of alfalfa root nodules, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 5273–5277.
[9] L.M. Zahn, C. Leebens-Mack, H. de Pamphils, G. Theissen, To B or not to B a Flower: the role of *DEFICIENS* and *GLOBOSA* orthologs in the evolution of the angiosperms, J. Hered. 96 (2005) 225–240.
[10] C. Wu, Q. Ma, K.M. Yam, M.Y. Cheung, Y. Xu, T. Han, H.M. Lam, K. Chong, *In situ* expression of the *GmNMH7* gene is photoperiod-dependent in a unique soybean (*Glycine max* [L.] Merr.) flowering reversion system, Planta 223 (2005) 725–735.
[11] W.P. Halford, V.C. Falco, B.M. Gebhardt, D.J. Carr, The inherent quantitative capacity of the reverse transcription-polymerase chain reaction, Anal. Biochem. 266 (1999) 181–191.
[12] Z. Li, H. Trick, Rapid method for high-quality RNA isolation from seed endosperm containing high levels of starch, BioTechniques 38 (2005) 872–876.
[13] P. Bauer, M. Crespi, J. Szècsi, L. Allison, M. Schultze, P. Ratet, E. Kondorosi, A. Kondorosi, Alfalfa *Enod12* genes are differentially regulated during nodule development by nod factors and *Rhizobium* invasion, Plant Physiol. 105 (1994) 585–592.
[14] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriphage T4, Nature 227 (1970) 680–685.

[15] J. Vasse, F. de Billy, S. Camut, G. Truchet, Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules, J. Bacteriol. 172 (1990) 4295–4306.
[16] K. Szczyglowsky, L. Amyot, Symbiosis, inventiveness by recruitment? Plant Physiol. 131 (2003) 935–940.
[17] K. Silverstain, M. Graham, K. VandenBosch, Novel paralogous gene families with potential function in legumes nodules seeds, Curr. Opin. Plant Biol. 9 (2006) 142–146.
[18] R.E. Macchiavelli, G. Brelles-Mariño, Nod factor-treated *Medicago truncatula* roots and seeds show an increased number of nodules when inoculated with a limiting population of *Sinorhizobium meliloti*, J. Exp. Bot. 55 (2004) 2635–2640.
[19] N.H. Markelz, D.E. Costich, T.P. Brutell, Photomorphogenic response in maize seedling development, Plant Physiol. 133 (2003) 1578–1591.
[20] J.A. Silveira, P. Clergeot, J.C. Quirion, H.P. Husson, A. Kondorosi, P. Ratet, Production of *Sinorhizobium meliloti nod* gene activator and repressor flavonoids from *Medicago sativa* roots, Mol. Plant Microb. Interact. 11 (1998) 784–794.
[21] A. Lalusin, K. Nishita, S.H. Kim, M. Ohta, T. Fujimura, A new MADS-box gene (*IbMADS10*) from sweet potato (*Ipomoea batatas* [L.] Lam) is involved in the accumulation of anthocyanin, Mol. Gen. Genom. 275 (2006) 44–54.
[22] M. Bey, K. Stüber, K. Fellenberg, Z. Schwarz-Sommer, H. Sommer, H. Saedler, S. Zachgo, Characterization of antirrhinum petal development and identification of target genes of the class B MADS box gene *DEFICIENS*, Plant Cell 16 (2004) 3197–3215.
[23] J.B. Glover, C. Martin, The role of petal cell shape and pigmentation in pollination success in *Antirrhinum majus*, Heredity 80 (1998) 778–784.
[24] T. Kurata, K. Okada, T. Wada, Intercellular movement of transcription factors, Curr. Opin. Plant Biol. 8 (2005) 600–605.
[25] A. Complainville, L. Brocard, I. Roberts, E. Dax, N. Sever, A. Kondorosi, S. Wolf, K. Oparka, M. Crespi, Nodule initiation involves the creation of a new symplasmic field in specific root cells of *Medicago* species, Plant Cell 15 (2003) 2778–2791.

Capítulo II

Estructura modular e interacciones proteína–proteína de los factores de transcripción con dominio MADS

Los recientes estudios filogenéticos en *Arabidopsis thaliana* han propuesto la existencia de dos linajes evolutivos representados por los genes MADS box tipo I y los tipo II (Álvarez-Buylla et al. 2000). Los genes MADS box tipo I tienen mayor homología con los factores de transcripción tipo SRF (Serum Response Factor) de animales y hongos, mientras que la secuencias de los tipo II están relacionada con MEF2 (Myocyte Enhancer Factor), también presentes en animales y hongos.

Muchas de las proteínas con dominio MADS de plantas tipo II poseen una estructura similar designada MIKC, por las iniciales de sus dominios característicos. En estas proteínas, el dominio MADS es la región más conservada y constituye el dominio de unión al ADN, el cual está compuesto de aproximadamente 60 aminoácidos. Este dominio se une a regiones específicas con la secuencia consenso $CC(A/T)_6GG$ llamadas cajas CArG (Pellegrini et al. 1995). El segundo dominio conservado es la caja K, de aproximadamente 70 aminoácidos, y se caracteriza por residuos hidrofóbicos espaciados regularmente. La estructura resultante es una hélice amfipática (similar a la queratina) que es responsable de la dimerización. Este dominio particular, al parecer, es una adquisición evolutiva exclusiva de las proteínas con dominio MADS de plantas, ya que está ausente en las proteínas MADS de animales y hongos (Álvarez-Buylla et al. 2000). El dominio intermedio o conector llamado I (del inglés *intermediate*) es de longitud variable, con un promedio de 30 residuos y se localiza entre la caja MADS y la caja K. Las proteínas MADS se unen al ADN como homo o heterodímeros y los determinantes moleculares que controlan la especificidad en la dimerización se distribuyen entre la caja MADS, la caja I y la caja K (Reichman et al. 1996). Los modelos de interacción más recientes proponen que la unión al ADN se consigue a través de la formación de complejos multiméricos, que podrían interactuar con un par de cajas CArG aproximándose gracias al doblamiento del ADN (Theissen & Seadler 2001). Por último, la región C-terminal es una secuencia de longitud variable y se cree que interviene en interacciones proteína–proteína actuando como un dominio de transactivación (Kaufmann et al. 2005).

La especificidad de los factores de transcripción por genes blanco puede ser potenciada por la unión cooperativa de varias proteínas; incluso, las interacciones con proteínas que no se unen al ADN o las modificaciones post-traduccionales pueden influir la regulación de la expresión genética. Algunos cofactores regulan la actividad de proteínas MADS sin requerir un dominio adicional de unión al ADN, como lo evidencian la interacción de MCM1 con proteínas que presentan homeodominios (Pramila et al. 2002). Por otra parte, las proteínas con dominio MADS pueden regular la transcripción sin unirse al ADN. Al actuar MEF2A sinérgicamente con proteínas bHLH durante la miogénesis, la regulación transcripcional puede requerir cajas CArG (dominios de unión de MEF2) o cajas E (dominios de unión bHLH/E12). La actividad de factores de transcripción con dominios MADS también puede ser regulada postranscripcionalmente. Por ejemplo, se ha sugerido que la fosforilación de MEF2A por la proteína p38 potencía la expresión de genes blanco (Zhao et al. 1999).

En plantas es posible distinguir entre interacciones con otras proteínas tipo MIKC y con cofactores no MADS. Sin embargo, existen escasas evidencias de cofactores diferentes a MADS que interaccionen con proteínas MIKC; OsMADS 18 –una proteína tipo-SEPALLATA de *Oryza sativa*– interacciona con proteínas NF-YB que pertenecen al grupo de factores de transcripción que se unen a cajas CCAAT (Masiero et al. 2002). En la región promotora de varios genes *AG* se han detectado sitios de unión de las proteínas que se unen a cajas CCAAT, además, este sitio puede ser importante para la expresión correcta de varios genes *AG in vivo* (Hong et al. 2003). La proteína MIP1 (del inglés MADS-box INTERACTING PROTEIN1) interacciona con las proteínas con dominio MADS de *Antirrhinum* (PLENA, FARINELLI, DEFH72 y DEFH200) en ensayos de doble híbrido. MIP1 contiene un motivo de cremallera de leucinas que es capaz de activar los genes reporteros en levaduras. Debido a que MIP1 contiene un posible péptido de localización nuclear, se ha especulado que confiere actividad transcripcional a proteínas con dominio MADS que carecen de dominios de activación transcripcional (Causier et al. 2003).

Las interacciones ternarias de proteínas con el dominio MADS se han estudiado extensivamente en mamíferos y levaduras (Messenguy & Dubois 2003). En mamíferos, las proteínas que pertenecen a las subfamilias SRF y MEF2 presentan en la región C-terminal de la caja MADS un dominio de 15 residuos requerida para la interacción con otras proteínas. En el caso de SRF, las superficie de unión con el factor de trasncripción Elk-1 se ha mapeado en la segunda hoja β localizada al final de la caja MADS. La región de OsMADS18 requerida para interactuar con OsNF-TB1 es equivalente al dominio utilizado por SRF, MEF2A y MCM1 para interactuar con otros polipéptidos; ésto indica que los dominios usados por proteínas MADS box para interactuar con proteínas no relacionadas está conservada entre mamíferos, plantas y levaduras (Masiero et al. 2002). Distintos experimentos

y datos cristalográficos del factor de transcripción SRF unido a cajas CArG enfocaron la atención hacia dos posibles sitios de fosforilación (T159 y S162) agrupados estrechamente en el motivo αI-rizo de la caja MADS el cual se ha conservado evolutivamente desde levaduras hasta humanos (Hassler M. & Richmond T. 2001) (fig. 1).

Recientemente se ha propuesto un modelo en el cual la fosforilación de la serina 162 en el motivo αI-rizo de SRF es un determinante clave en la regulación de la conmutación entre replicación celular y diferenciación. En este sistema la caja MADS actúa como un nexo regulador que media la interacción entre una miríada de cofactores transcripcionales donde su fosforilación diferencial especificaría directamente la formación de complejos transcripcionales que determinarían de manera diferencial los genes necesarios para la diferenciación o la proliferación (Iyer et al. 2006). La interacción de SRF con distintos cofactores (Nkx2-5, GATA4, LIM, CRP2, miocardina y factores Ets) es mediante el motivo αI rizo. Todos estos cofactores miogénicos trans-activan a SRF indicando que esta región es un sitio importante para la recepción de señales intracelulares que favorecen que SRF reclute cofactores específicos a sus respectivos sitios de unión al ADN (Chang et al. 2003; Wang et al. 2004; Sepúlveda et al. 2002) (fig. 1)

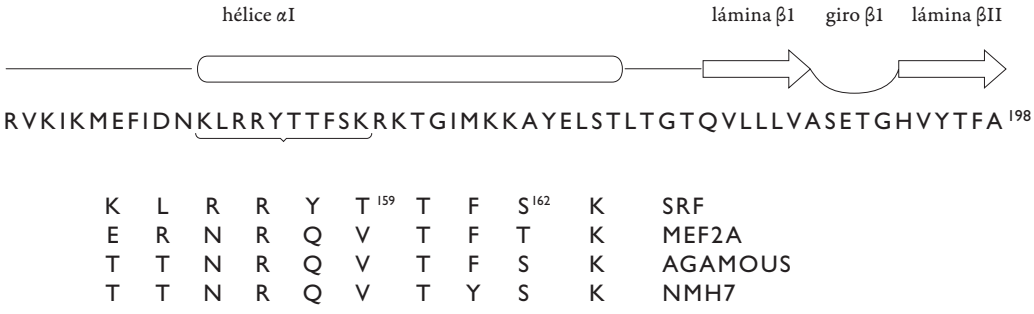

Fig. 1: Estructura del dominio MADS de SRF (aa 143-198) indicando la estructura secundaria. Se indica la secuencia ubicada (corchete) en el motivo αI rizo que contiene los residuos que forsforilados en SRF (T159, S162) y que median la interacción con cofactores tipo no MADS. También se muestran alineadas las secuencias correspondientes a MEF2A con la región empleada para aislar los interactores de AGAMOUS (Gamboa et al. 2001) y de NMH7 (Páez-Valencia et al. 2008 b).

Muchas de las interacciones proteína–proteína en los eventos de señalización son mediadas frecuentemente por secuencias cortas, las cuales interaccionan con dominios de unión a péptidos. El método más directo para determinar qué proteínas interaccionan con la secuencia de interés, es emplearlo como cebo (*bait*) en ensayos de afinidad (*pulldown*) seguido por la detección de las proteínas que interactúan con las secuencia inmovilizada (Shulze & Mann 2004). Esta aproximación metodológica se empleó para aislar proteínas que interactúan con el factor de transcripción AGAMOUS durante el desarrollo floral de *Arabidopsis thaliana*. La secuencia empleada como ligando inmovilizado posee motivos que median interacciones proteína–proteína, específicamente un motivo de fosforilación y un sitio de reconocimiento de proteínas fosfatasas. Así se logró aislar una proteína con mo-

tivos repetidos ricos en leucina (FLOR1) y una proteína con actividad de fosfatasa VSP (del inglés Vegetative Storage Protein), interacción que se corroboró por ensayos de doble híbrido e inmunoprecipitación (Gamboa et al. 2001).

El factor de transcripción con dominio MADS, NMH7, se expresa en flores y nódulos de *Medicago sativa,* lo que sugiere su participación en el desarrollo del nódulo (Heard & Dunn 1995). Sin embargo, usando RT-PCR, inmunodetección e inmunolocalización durante la primera parte del presente proyecto de investigación demostramos que NMH7 se expresa también en semillas, cotiledones y en la raíz primaria de plántulas no inoculadas. La presencia de NMH7 en etapas previas a la colonización por parte de las bacterias, sugiere que esta proteína está involucrada en eventos no simbióticos, o bien, podría regular programas de desarrollo relacionados con la colonización bacteriana (Páez-Valencia et al. 2008 b). Como primera aproximación para entender la función de NMH7 en plántulas de *Medicago sativa* que crecen en déficit de N y en etapas previas al desarrollo del nódulo, es necesario identificar a las proteínas que interactúan con NMH7 como posibles reguladores. Se utilizó una columna de afinidad con decapéptido derivado del domino MADS de NMH7 que contiene el motivo RQXXY (fig. 1) para identificar proteínas candidatas a interactuar con NMH7 regulando su función. Una vez aisladas e identificadas las proteínas que reconocen el decapéptido derivado del dominio MADS de NMH7, fue menester realizar pruebas subsidiarias de interacción como ensayos recíprocos de *pulldown* e inmunoprecipitación para corroborar que la interacción mostrada en la columna de afinidad puede ocurrir *in planta*. Por otra parte, es necesario que las proteínas que interactúan con NMH7 se localicen en los mismos tipos y compartimentos celulares durante el desarrollo. Por lo tanto, los estudios de co-localización permitieron determinar espacial y temporalmente los compartimentos celulares donde podría ocurrir la interacción entre NMH7 y la(s) proteína(s) interactora(s).

Restultados

Artículo:

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Identification of Fructose-1,6-bisphosphate aldolase cytosolic class I as an NMH7 MADS domain associated protein

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ABSTRACT

We are interested in identifying proteins that interact with the MADS domain protein NMH7 of *Medicago sativa*. We use an affinity column with a synthetic peptide derived from the MADS domain of NMH7 which has been reported to mediate protein–protein interaction with non-MADS domain interacting proteins. We identified ~40 and ~80 kDa specifically bound proteins as the monomeric and dimeric forms of Fructose-1,6-bisphosphate aldolase cytosolic class I. NiNTA pull down assays revealed that K- and C-terminus regions of NMH7 are not required for the interaction with aldolase. Aldolase enzymatic activity is not required for the interaction with NMH7. NMH7 and aldolase were coimmunoprecipitated from non-inoculated seed and seedlings extracts. Colocalization studies using confocal microscopy showed that aldolase and NMH7 are localized in the cytoplasm and the nucleus of the cortical cells. These data together show that *M. sativa* aldolase is a novel MADS domain binding protein, and suggest a broader functional repertory for this enzyme, as has been proposed for other glycolytic enzymes.

Transcription factors belonging to the MADS box family contain a highly conserved DNA-binding domain, which was originally identified by comparing four transcription factors: the yeast MCM1, AG and DEF from plants, and the human serum response factor (SRF). Different mechanisms including phosphorylation, the chromatin structure, and specific interaction to different proteins to form complexes have been determined for the specific regulation of downstream genes in differing cell types [1]. It has recently been demonstrated that the presence of SFR cofactors is the main factor involved in SRF DNA-binding specificity [2]. The interaction between the cofactors and the MADS transcription factors is mainly through the MADS domain.

MADS box genes perform different functions during plant development including nodule development in which the host tissues and the bacterial microsymbiont develop in response to each other to form a specialized tissue that maintains an environment in which nitrogen fixation can occur. *NMH7* is among several members of the MADS box genes expressed in nodules of the legume plant *Medicago sativa*, suggesting an important role of these transcription factors in nodule development [3]. The presence of NMH7 in non-inoculated seeds, cotyledon seedling and primary roots suggests that this protein is involved in non-symbiotic events, or that it is involved in developmental programs related to bacterial colonization in seedlings [4]. To investigate the role of NMH7 in *M. sativa* seedlings growing under nitrogen limitation prior to nodule development, we wish to identify different proteins as its potential regulators. In the present study we use an affinity column, with a decapeptide derivate from the MADS domain of NMH7 containing the RXXTY motif, to isolate putative proteins that interact with the NMH7 transcription factor in non-inoculated seedlings of *M. sativa*. Fructose-1,6-bisphosphate aldolase cytosolic class I (aldolase) was identified as a putative binding NMH7 partner. We confirmed the interaction between NMH7 and aldolase *in vitro* by using a recombinant protein in reciprocal pull down experiments. *In vivo* interaction of these proteins was detected using immunoprecipitation, Western blotting and colocalization analysis. This is the first report to describe a glycolytic enzyme that interacts directly with a plant MADS domain transcription factor.

Materials and methods

Plant material and growth conditions. Medicago sativa seeds germination and inoculation were performed as described in Páez-Valencia et al. [4].

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Peptide synthesis and pulldown. The oligopeptide synthesis (TTNRQVTYSK) was done by INVITROGENE, and the peptide was immobilized in Affigel10 according to the manufacturer's instructions (Bio-Rad). Peptide pull down assay using the affinity column was performed as described in Gamboa et al. [5]. The non-decapeptide coupled resin was used as negative control.

Protein identification. The fractions obtained in the affinity chromatography assays were analyzed on denaturing 12% SDS–PAGE gels and stained according to Vorum's silver stain, compatible with in-gel digest for sequencing. The bands of interest were extracted from the gels and sent to the Proteomics Platform of the Eastern Genomics Center, Quebec, Canada, where the in-gel digest and mass spectrometry experiments were performed. Tryptic digestion was performed according to the method of Shevchenko et al. [6] and Havlis et al. [7]. Peptide samples were separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nano/LC) and were analyzed by electrospray mass spectrometry (ES-MS/MS). All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2.0). Scaffold (version Scaffold-01_07_00, proteome Software, Inc. Portland Oregon, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they were established at greater than 95.0% probability, as specified by the Peptide Prophet algorithm [8]. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [9].

Medicago sativa Fructose-1,6-bisphosphate aldolase cDNA cloning. Plant Fructose-1,6-bisphosphate aldolase cytosolic class I homologs were searched in public databases with BLASTN application. The most significant nucleotide sequences were chosen to design the oligonucleotide primers. Total RNA was purified with TRIZOL (INVITROGENE) and cDNA was synthesized using the Super Script II (INVITROGENE). The primer sequences were: ALDO F 5′CCTCAT GACGAACTTCCAAGAG3′ and ALDO R 5′AGATCTGTAGTCCTTAAC3′. Cloned PCR products into TOPO vectors were sequenced.

Preparation of histidine-tagged recombinant proteins. The construction and expression of NMH7-KC $(His)_6$ and NMH7-MIKC expression has been described previously [4]. For ALDO (His)6 construction, the aldolase coding region was amplified using the primer sense ALDO F 5′GGATCCACCGAACTTCAAGAG3′ and antisense 5′GGATCCGTAGTCCTTAACATG3′, adding *BamH1* at both extremes (underlined sequence). The product was cloned into a pQE30 vector (QIAGEN) and expression was done under manufacturer's instructions. The purified proteins were analyzed by SDS–PAGE and were developed with silver staining reagent (GE Healthcare) to verify the quality of purification. The heterologous overproduction of *M. sativa* aldolase is problematic because of the usage of codons that are rarely used in *E. coli*, particularly arginine (AGA/AGG) in the sequence. The expression of pQE30 resulted in the overproduction of truncated derivates of ~29 kDa. This phenomenon has been reported for heterologous expression of eukaryotic protein in *Escherichia coli* [10]. His-tagged recombinant NMH7-MIKC and NMH7-KC were purified from *E. coli* transformed with pQE60/NMH7-MIKC and pQE60/NMH7-KC constructs, respectively. The cells were centrifuged and the pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole), sonicated for 2 min on ice, and shaken for 30 min at room temperature. The extracts were centrifuged at 15,000*g* for 20 min. One milliliter of NiNTA-Agarose affinity resin (QIAGEN) was then added to the supernatant, and the mixture was shaken at room temperature for 1 h. The mixture was loaded into a plastic column (Bio-Rad), and was washed with 40 mL of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM Imidazole) and then equilibrated with 20 mL of equilibrium buffer (20 mM Tris−HCl, pH 5.5, 2 mM $MgCl_2$, 1 mM PMSF). At the same time, a crude extract of seedling was prepared with 30 mL of extraction buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM PMSF). His-tagged protein resin was treated with the crude extracts from *M. sativa* seedlings and incubated for 60 min at room temperature with gentle shaking. The NiNTA resins were recovered, transferred to a column, and washed with 40 mL of equilibrium buffer. Bound proteins were eluted with equilibration buffer with 1 M NaCl and were concentrated in an AMICON filter at 4 °C. The bound proteins were detected by immunoblotting using anti-aldolase antibodies produced in mice. For reciprocal pull down, His-tagged aldolase bound to NiNTA resin was incubated with crude extract of seedlings. Protein purification was performed as described above. The eluted proteins were detected by immunoblotting using antibodies raised against the KC domains of NMH7 protein.

Production of antibodies and Western blot analysis. The production of rabbit polyclonal antibodies against the KC domains of pro-

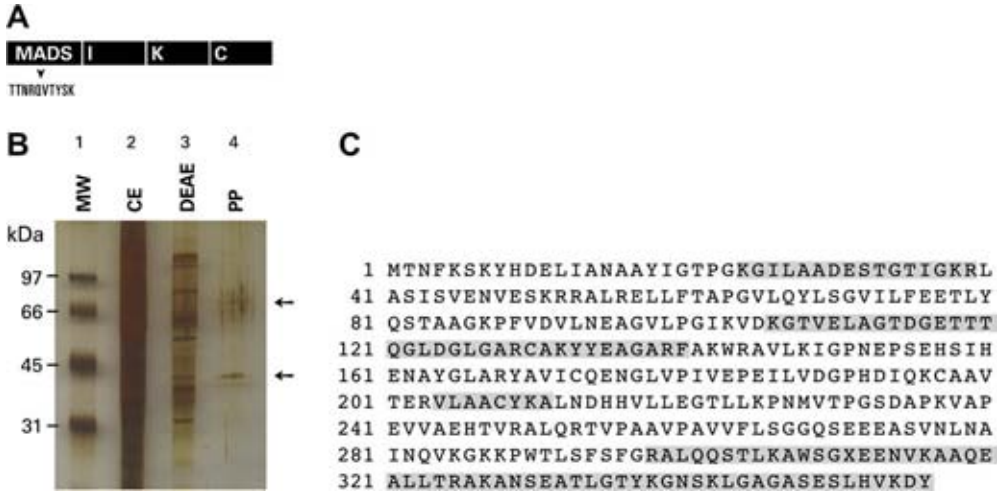

Fig. 1. Identification of Fructose-1,6-bisphosphate aldolase cytosolic class I as an NMH7 associated protein. (A) Modular structure of NMH7 showing the four canonical domains of MICK type MADS transcription factors. The peptide used in the affinity assay is indicated. (B) SDS–PAGE of the complex that interacts with TTNRQVTYSK. Lane 1, molecular weight marker; lane 2, crude extract from *M. sativa* seedlings; lane 3, DEAE-Sephacel flow through; lane 4, eluted proteins. The two bands (40–80 kDa) were identified as Fructose-1,6-bisphosphate aldolase cytosolic class I, corresponding to the monomeric and the SDS-resistant dimeric forms, respectively (indicated by arrows). (C) Deduced amino acid sequence of *M. sativa* Fructose-1,6-bisphosphate aldolase cytosolic class I. The peptides identified by LC–MS/MS are indicated (shaded sequences).

tein has been described elsewhere [4]. For the complete aldolase protein, a polyclonal antibody was produced using the same protocol [4]. To produce a specific peptide antibody, we followed the methodological approach described by Anderson et al. [11]. In outline, the aldolase cytosolic isozyme specific peptide (H-TQGLDGLG-ARCK-OH) was synthesized. This peptide was conjugated to keyhole limpet haemocyanine carrier protein with glutaraldehyde, and the coupled peptide was injected intraperitoneally into eight mice. Preimmune and immune sera were collected and used without further purification.

Western blotting analyses were done as previously described [4].

Immunoprecipitation and immunoblotting. Seeds and 3-day-old seedlings were homogenized in an extraction buffer containing 50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 300 mM NaCl, 0.1% Triton X-100 and complete protease inhibitor mixture (ROCHE), which was then centrifuged at 20,000*g*. The protein concentration was determined by dye binding assay (Bradford Coomassie Reagent, AMRESCO). Protein samples (1 mg of protein) were incubated with the primary antibody or preimmune serum at 4 °C for 6 h, followed by incubation with agarose-protein G under the same conditions. The beads were suspended in SDS–PAGE buffer, and the samples underwent Western analysis against appropriate antibodies.

Double immunofluorescence staining. Tissue embedding and sectioning were performed as described previously [4]. Slides with tissue sections were deparaffinized with xylene and were rehydrated through a graded ethanol series. After deparaffination and hydration, the slides were washed in PBS. To recover antigenicity, histological sections were immersed in Antigen Retrieval Citra Plus solution (BioGenex HK080-5K) and autoclaved for 5 min. The slides were pre-treated with Power Universal Reagent (BioGenex HK085-5K) to reduce non-specific binding, and were incubated with the primary antibodies (rabbit NMH7-KC and mouse anti-aldolase) at a dilution of 1:200 in PBS Tween or preimmune sera for 1 h at room temperature. Three rinses with PBS were followed by incubation with FITC-conjugate goat anti-mouse secondary antibody and Texas Red-conjugated anti-Rabbit secondary antibody. The coverslips were washed thoroughly and mounted. The immunostained tissues were observed at room temperature by laser confocal microscopy.

Results and discussion

Identification of Fructose-1,6-bisphosphate aldolase cytosolic class I as a possible NMH7 interacting protein in non-inoculated seedlings of M. sativa

To search for protein binding partners of NMH7 in *M. sativa* seedlings, we used an affinity column with a synthetic peptide derived from the MADS domain sequence, which is reportedly involved in protein–protein interactions between MADS domain proteins and non-MADS cofactors. This motif has previously been used to isolate an LRR protein (FLOR1) and an acid phosphatase protein in *Arabidopsis thaliana* flowers [5].

The protein fraction eluted developed two bands of ~40 and ~80 kDa on the silver-stained SDS–PAGE gel (Fig. 1B). Sequence analysis of the two bands by LC/MS/MS gave 10 peptides that matched Fructose-1,6-bisphosphate aldolase cytosolic class I (D-Fructose-1,6-bisphosphate D-glyceraldehyde-3-P-lyase E.C. 4.1.2.1.3, Fig. 1C). The 40 kDa band is the monomeric form of the enzyme, and 80 kDa corresponds to the dimeric form. These forms are fully active although this enzyme has a highly conserved tetrameric structure. 80 kDa band corresponds to a dimeric SDS-resistant complex as it was described for the wild-type enzyme [12].

To confirm the *in vitro* interaction of the NMH7 MADS domain and aldolase, we performed pull down analysis using an affinity column, either with the complete NMH7 protein (NMH7-MIKC $(His)_6$) and the truncated NMH7 protein (NMH7-KC $(His)_6$). Aldolase interacts specifically with the MADS domain; aldolase was detected with specific antibodies in the NMH7-MIKC column eluted samples, but no signal was detected in samples eluted from the NMH7-KC column (Fig. 2B and C). These results confirm that the MADS domain is involved in the interaction of NMH7 with aldolase, as it was found for the OsMADS 18 interaction with OsNF-YB1, which is a non-MADS box transcription factor [13].

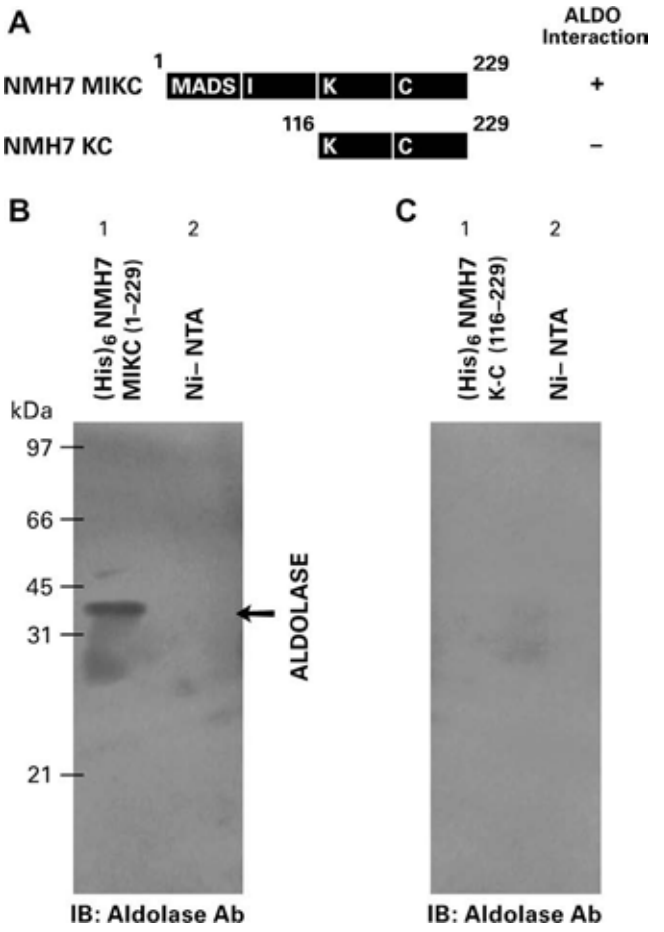

Fig. 2. Aldolase binding to NMH7 MADS domain. (A) Schematic representation of (His 6) tagged NMH7 domains and their ability to bind aldolase. The rightmost column indicates whether an interaction was observed. (B) Aldolase interacts with the complete NMH7-MIKC. Lane 1: An aliquot of the eluted proteins that interact with complete NMH7 (MIKC) was subjected to SDS–PAGE (12%) and aldolase was detected using the antibody raised against isoform specific peptide. Lane 2: Negative control; aldolase was not detected from the eluted protein fraction using NiNTA resin. (C) Aldolase does not interact with the NMH7-KC domain. Lane 1: An aliquot of the eluted proteins that interact with NMH7-KC was subjected to SDS–PAGE (12%). No aldolase signal was detected by Western blotting (IB) using the mouse antibody (ab). Lane 2: Negative control; aldolase was not detected from the eluted protein fraction using NiNTA resin Three independent experiments were performed and all gave similar results.

Fructose-1,6-bisphoshate aldolase catalyzes the reversible cleavage of FBP into glyceraldehyde-3-P and dihydroxyacetone [14]. Aldolase not only plays a key role in glycolysis, but also binds to macromolecules unrelated to glycolysis including actin, tubuline and RNA. Consequently, the aldolase may be used for other functions in cells, in which respect it has been described as moonlighting [15–16]. Band 3, the major intrinsic membrane protein of red cells and the principal Cl^-/HCO^{3-} exchanger, has a cytoplasmic domain that is the anchoring site for several glycolytic enzymes such aldolase. The N-terminal 11 amino acids which are the critical res-

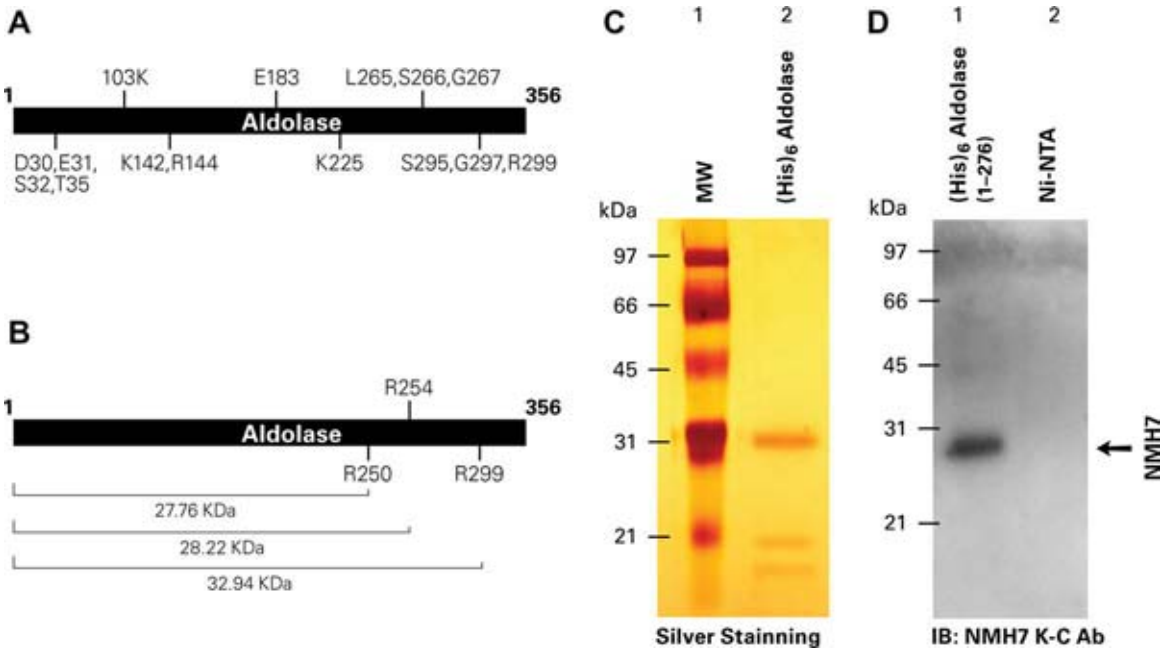

Fig. 3. Binding of the NMH7 to aldolase *in vitro*. (A) Schematic representation of aldolase showing the residues involved in the catalytic function. (B) Schematic representation of aldolase showing the Arginine (R) residues that are codified AGA/AGG. The rare usage of these codons by bacteria resulted in a truncated recombinant protein with less catalytic residues. The three possible products are shown. (C) Lane 1: Molecular weights. Lane 2: The main band corresponds to a truncated product of ~29 kDa which is catalytically inactive. (D) NMH7 interaction with the 29 kDa aldolase. Lane 1: Proteins bound to truncated aldolase were recovered and analyzed by Western blotting (IB) NMH7 was detected with anti NMH7 antibody (ab). Lane 2: Negative control. Three independent experiments were performed and all gave similar results.

idues for aldolase binding include QXXY, a motif that is phosphorylated/dephosphorylated by protein tyrosine kinase Syk and tyrosine phosphatases, regulating this binding [17]. This motif could also be involved in the aldolase binding to NMH7, since the peptide used in the affinity column contains the QXXY sequence. Our results confirm that the use of synthetic modified or unmodified peptides to find direct protein interaction partners is a powerful way to search for interactors and dissect the role of certain motifs during signaling events.

Aldolase enzymatic activity is not required for its interaction with NMH7

An important feature of moonlighting proteins is that the enzymatic activity is not involved in the alternative function. According to recent crystallographic studies, the highly stable structure of aldolase with an independent active site suggests that this protein has evolved as a multimeric scaffold protein to perform other functions without catalytic activity [18].

NMH7 interacts with the truncated form of aldolase with no activity (Fig. 3A–D). Our results show that aldolase catalytic activity is not necessary for interaction with the NMH7, as it was found for the aldolase mediated V-ATPase assembly [19]. These findings indicate that *M. sativa* aldolase interacts directly and selectively with NMH7.

NMH7-aldolase in vivo interaction in seeds and seedlings of M. sativa

To determine whether NMH7 and aldolase interact *in vivo*, we carried out immunoprecipitation of aldolase from seeds and seedlings, using an antibody raised against a recombinant truncated form of aldolase. As shown in Fig. 4D, specific aldolase antibodies coimmunoprecipitated NMH7, which was detected by Western blotting using a specific antibody raised against the KC domain of NMH7, whereas the control rabbit preimmune sera did not precipitate. A reciprocal coimmunopreciptation experiment was performed; NMH7-specific antibody coimmunoprecipitated aldolase, which was detected by Western blotting using a specific anti-aldolase (Fig. 4E). Thus, NMH7 MADS domain transcription factor is likely to form a complex with aldolase in seeds and seedlings.

NHM7 and aldolase colocalize in the nucleus

We used specific antibodies against aldolase and NMH7, which were subsequently detected by secondary antibodies conjugated to FITC (green channel) and Red Texas (red channel), respectively. In 4-day-old seedlings, NMH7 and aldolase colocalized in the cytoplasm and the nucleus of cortical cells of the primary root (Fig. 4A1–A3). In the cortex inner layer of nodules, both NMH7 and aldolase have a punctuated nuclear distribution of speckled form (Fig. 4B1–B3). The two aldolase isoenzymes described in *Pisum sativum* are also present in the nucleus and were detected over the euchromatin and the nucleoli, suggesting a role in gene expression [11]. A similar pattern has previously been reported for mammalian aldolase in the cardiomyocite nuclei [20]. The metabolic enzymes could act as a sensor of the metabolic condition of the cell. Characterization of the multiple component coactivator complex essential for Histone H2B transcription during the S phase identifies a nuclear glyceraldehyde-3-phosphate dehydrogenase. The histone *H2B* promoter is modulated by NAD+/NADH, and GADPH may act by sensing and transducing the cellular redox state [21].

Recent studies have found a characteristic metabolic "marker pattern" in the symbiosis between leguminous plants and rhizobia. Comparison of the relative metabolite abundances in nodules versus nitrogen starved *M. sativa* roots showed that the ratios of different amino acids and phosphorylated metabolites increase [22]. Glucose increases aldolase and V-ATPase interaction in yeast cells, and aldolase substrates disrupt the actin–aldolase–GLUT4 complex involved in GLUT4 vesicle trafficking [19,23]. These reports suggest that aldolase acts as a signaling receptor of the metabolic status of the cell. It is therefore plausible that, in *M. sativa,* aldolase links the

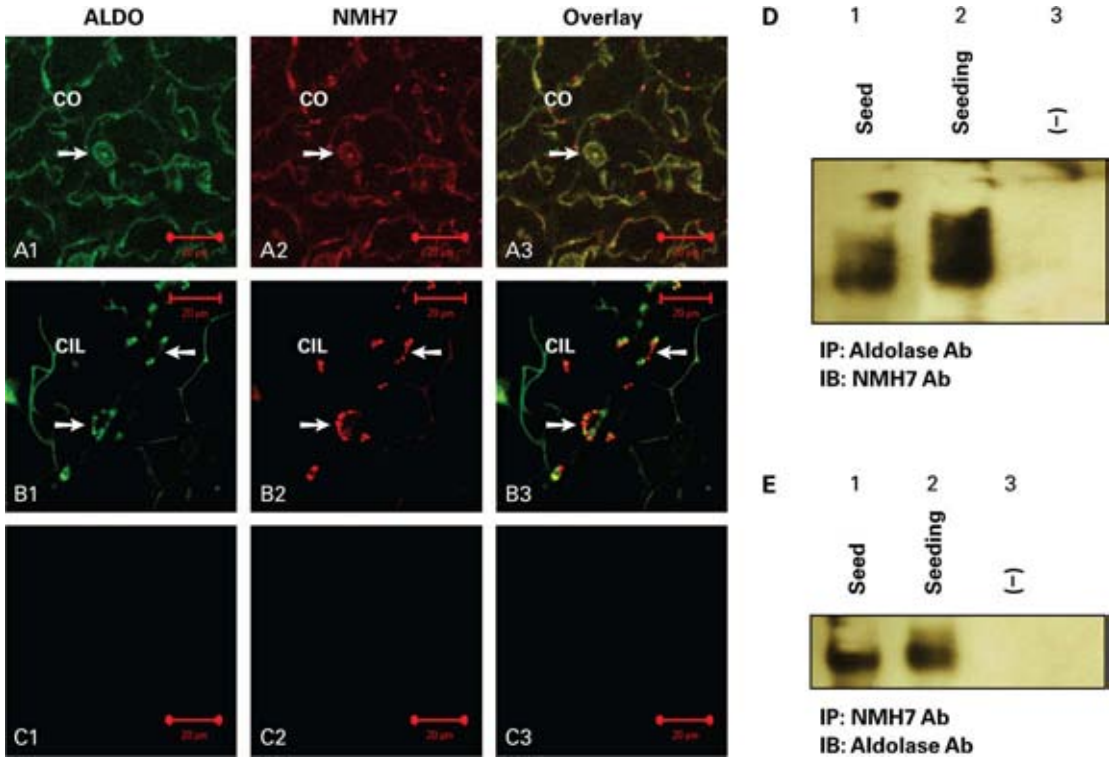

Fig. 4. *In vivo* association of NMH7 with aldolase. (A) Colocalization of NMH7 and aldolase in seedlings of *M. sativa*. Tissues embedding sections were processed for confocal immunolocalization using antisera specific for aldolase and NMH7, followed by secondary antibodies labeled with FITC (green channel) and Red Texas (red channel), respectively. Transversal sections of 4-day-old seedlings were stained for aldolase (A1) and NMH7 (A2); the merged image (yellow signal) shows a nucleo-cytoplasmic localization of NMH7 and aldolase in the cortical cell of the root (A3). (B1–B3) A region of the apical region of the *M. sativa* nodules is shown, containing the cortex inner layer and a nuclear colocalization with speckle like structure (arrows). (C1–C3) As negative control the sections were incubated with preimmune sera; no signal was detected. co, cortical cells; cil, cortex inner layer. A1–A3; B1–B3; C1–C3; scale bars = 20 μm. Three independent experiments were performed and all gave similar results. (B) Coimmunoprecipitation of NMH7 and aldolase from *M. sativa* seedlings and seeds using aldolase antibodies. A crude extract from non-inoculated *M. sativa* seeds (lane 1) and seedlings (lane 2) was subjected to immunoprecipitation (IP) using aldolase rabbit antibodies (Ab). The immunoprecipitates were analyzed by Western blotting with antibodies raised against the KC domain of NMH7. As negative control, crude extract from seedlings was incubated with rabbit preimmune sera (lane 3); no signal was detected. (C) *Reciprocal coimmunoprecipitation.* Crude extracts from non-inoculated *M. sativa* seeds (lane 1) and seedlings (lane 2) were subjected to immunoprecipitation using antibodies raised against the KC domain from NMH7. The immunoprecipitates were analyzed by Western blotting with the aldolase peptide mouse antibody. As negative control, the crude extract was incubated with rabbit preimmune sera (lane 3). These data are representative of three independent experiments, with similar results.

metabolic status with the central developmental network. Our findings open new possibilities for examining the roles of aldolase and other metabolic enzymes in the plant nucleus. A new challenge is to determine how aldolase and NMH7 are targeted to the nucleus to form complexes with other components, and to understand the combinatorial control of transcription in response to environmental cues that regulate plant development.

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References

[1] F. Messenguy, E. Dubois, Role of MADS box proteins and their cofactors in combinatorial control of gene expression and cell development, Gene 16 (2003) 1–21.
[2] S.J. Cooper, N.D. Trinklein, L. Nguyen, R.M. Myers, Serum response factor binding sites differ in three human cell types, Genome Res. 17 (2007) 136–144.
[3] J.C. Zucchero, J.C. Caspi, K. Dunn, Ngl9: a third MADS box gene expressed in alfalfa root nodules, Mol. Plant Microbe Interact. 14 (2001) 1463–1467.
[4] J. Páez-Valencia, C. Sánchez-Gómez, P. Valencia-Mayoral, A. Contreras-Ramos, I. Hernández-Lucas, A. Orozco-Segovia, A. Gamboa-deBuen, Localization of the MADS domain transcription factor NMH7 during seed, seedling and nodule development of *Medicago sativa*, Plant Sci. 175 (2008) 596–603.
[5] A. Gamboa, J. Páez-Valencia, F. Acevedo, L. Vazquez-Moreno, R.E. Alvarez-Buylla, Floral Transcription Factor AGAMOUS Interacts in vitro with a leucine-rich repeat and an acid phosphatase protein complex, Biochem. Biophys. Res. Commun. 288 (2001) 1018–1026.
[6] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels, Anal. Chem. 68 (1996) 850–858.
[7] J. Havlis, H. Thomas, M. Sebela, A. Shevchenko, Fast-response proteomics by accelerated in-gel digestion of proteins, Anal. Chem. 75 (2003) 1300–1306.
[8] A. Keller, A.I. Nesvizhskii, E. Kolker, R. Aebersold, Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search, Anal. Chem. 74 (2002) 5383–5392.
[9] A.I. Nesvizhskii, A. Keller, E. Kolker, R. Aebersold, A statistical model for identifying proteins by tandem mass spectrometry, Anal. Chem. 75 (2003) 4646–4658.
[10] T.S. Matambo, O.O. Odunuga, A. Boshoff, G.L. Blatch, Overproduction, purification, and characterization of the *Plasmodium falciparum* heat shock protein 70, Protein Expr. Purif. 33 (2004) 214–222.
[11] L.E. Anderson, M.R. Ringenberg, V.K. Brown, A.A. Carol, Both chloroplastic and cytosolic phosphofructoaldolase isozymes are present in the pea leaf nucleus, Protoplasma 225 (2005) 235–242.
[12] J. Gerschitz, R. Rudolph, R. Jaenicke, Kinetics of reactivation of rabbit muscle aldolase after denaturation and dissociation in various solvent media, Biophys. Struct. Mech. 3 (1977) 291–302.
[13] S. Masiero, C. Imbriano, F. Ravasio, R. Favaro, N. Pelucchi, M.S. Gorla, R. Mantovani, L. Colombo, M.M. Kater, Ternary complex formation between MADS-box transcription factors and the histone fold Protein NF-YB, J. Biol. Chem. 277 (2002) 26429–26435.
[14] T. Gefflaut, C. Blonski, J. Perie, M. Willson, Class I aldolases: substrate specificity, mechanism, inhibitors and structural aspects, Prog. Biophys. Mol. Biol. 63 (1995) 301–340.
[15] J. Wang, A.J. Morris, D.R. Tolan, L. Pagliaro, The molecular nature of the F-actin binding activity of aldolase revealed with site-directed mutants, J. Biol. Chem. 271 (1996) 6861–6865.

[16] R. Cañete-Soler, K.S. Reddy, D.R. Tolan, J. Zhai, Aldolases A and C are ribonucleolytic components of a neuronal complex that regulates the stability of the light-neurofilament mRNA, J. Neurosci. 27 (2005) 4353–4364.
[17] L. Bordin, A.M. Brunati, A. Donella-Deana, B. Baggio, A. Toninello, G. Clari, Band 3 is an anchor protein and a target for SHP-2 tyrosyne phosphatase in human erythrocytes, Blood 100 (2002) 276–282.
[18] M. Sherawat, D.R. Tolan, K.N. Allen, Structure of a rabbit muscle fructose-1,6-bisphosphate aldolase A dimer variant, Acta Crystallogr. D: Biol. Crystallogr. 64 (2008) 543–550.
[19] M. Lu, D. Ammar, H. Ives, F. Albrecht, S.L. Gluck, Physical interaction between aldolase and vacuolar H^+ ATPase is essential for the assembly and activity of the proton pump, J. Biol. Chem. 282 (2007) 24495–24503.
[20] P. Mamczur, D. Dus, A. Dzugaj, Colocalization of aldolase and FBPase in cytoplasm and nucleus of cardiomyocytes, Cell Biol. Int. 31 (2007) 1122–1130.
[21] L. Zheng, R.G. Roeder, Y. Luo, S phase activation of the Histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component, Cell 114 (2003) 255–266.
[22] A. Barsch, V. Tellström, T. Patschlkowski, H. Küster, K. Niehaus, Metabolite profiles of nodulated alfalfa plants indicate that distinct stages of nodules organogenesis are accompanied by global physiological adaptations, Mol. Plant Microbe Interact. 19 (2006) 998–1013.
[23] A.W. Kao, Y. Noda, J.H. Johnson, J.E. Pessin, A.R. Saltiel, Aldolase mediates the association of F-actine with the insuline-responsive glucose transporter GLUT-4, J. Biol. Chem. 274 (1999) 17742–17747.

Capítulo III

Regulación nutricional de los factores de transcripción

Diversos estudios han descrito los múltiples cambios que ocurren en la expresión genética tras la privación de un nutriente individual (Maathuis et al. 2003; Wasaki et al. 2003; Wintz et al. 2003; Armengaud et al. 2004; Hirai et al. 2004). Sin embargo, otras investigaciones sugieren una interacción *cross-talk* entre las rutas de señalización por la privación de fósforo/potasio/hierro y nitrógeno/fósforo/potasio (Wang et al. 2002; Hammond et al. 2003). Este *cross-talk* también es sugerido por estudios con microarreglos, los cuales muestran que la privación de un solo nutriente permite la inducción de un amplio número de transportadores de iones (Franco Zorrilla et al. 2004; Hammond et al. 2003). Por ejemplo, los genes que codifican para tres transportadores de NO_3^- son inducidos por la limitación de potasio (Armengaud et al. 2004). Invariablemente, los factores de transcripción se encuentran entre los genes regulados diferencialmente, implicándolos en la regulación específica de procesos asociados al desarrollo o en la respuesta al ambiente biótico y abiótico (Udvardi et al. 2007). Recientemente se aisló una subfamilia de genes que codifican factores de transcripción R2R3-MYB cuya expresión es regulada positivamente bajo condiciones limitantes de N; por otra parte, el análisis de la expresión de uno de estos genes, MYB101, mostró que es regulado por NO_3^- y podría estar involucrado en la biosíntesis de flavonoides en respuesta a la deficiencia de N (Miyake et al. 2003).

En *Arabidopsis thaliana,* el gene *ANR1* ha sido identificado como componente de una ruta de señalización que regula el crecimiento de raíces laterales en respuesta a cambios en el abastecimiento externo de NO_3^- (Zhang & Forde 1998). *ANR1* codifica a un miembro de la familia de factores de transcripción MADS box cuya expresión es estimulada por la ausencia de NO_3^- en el medio e inhibida por el reabastecimiento de N. Otros genes MADS box que también responden de manera similar a *ANR1* son *AGL16, AGL21, AGL14, AGL19, AGL26, AGL56* y *SOC1* (Gan et al. 2005), *SOC1* también responde a los cambios en el suministro de P y Azufre (S), lo que sugiere la intervención de los genes MADS box en la regulación nutricional del desarrollo de raíces laterales (Gan et al. 2005).

El desarrollo del nódulo es un proceso estrechamente controlado por un mecanismo interno llamado autorregulación, pero el establecimiento de una simbiosis funcional también es regulado por el N del suelo (principalmente NO_3^-); específicamente, la aplicación de N inhibe todas las fases del proceso de nodulación incluyendo la colonización por parte de la bacteria, el desarrollo del nódulo y la fijación del N_2. En leguminosas, el P es el nutriente limitante y su deficiencia puede disminuir la nodulación y la fijación del N_2 (Tan et al. 2001). También se ha mostrado que en altas concentraciones de N, el P tiene un efecto antagónico sobre el efecto inhibidor del N en la nodulación (Huss-Dannell 2000).

La localización de NMH7 en las células corticales de las plántulas no inoculadas de *Medicago sativa,* hacen suponer que NMH7 está involucrado en eventos de desarrollo relacionados con la colonización bacteriana o bien, sensar las condiciones ambientales para activar una serie de genes blanco que conlleven al establecimiento de la simbiosis. El papel de NMH7 como proteína sensora es perfilado por la interacción de este factor de transcripción con la fructosa-1,6-bisfosfato aldolasa citosólica clase I en plántulas crecidas en medio libre de N y su co-localización en el núcleo de las células de la capa interna del córtex del nódulo simbiótico (Páez-Valencia et al. 2008). Para poder tener una comprensión integral de la posible función reguladora del factor de transcripción NMH7 en plántulas y nódulos, en el presente trabajo se planteó: (1) investigar la regulación nutricional de NMH7 analizando cómo la acción individual y conjunta del N y P afectan la expresión de NMH7 en plántulas de *Medicago sativa,* (2) determinar si la localización subcelular de NMH7 es dependiente del estado nutricional de la planta y (3) analizar la relación entre la inhibición de la nodulación promovida por NO_3^- y la expresión y localización de NMH7.

Resultados

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Nutritional regulation of MADS domain transcription factor NMH7: expression and subcellular localization imply it integrates environmental signals with plant development

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ABSTRACT

We investigated the responsiveness of NMH7 to nitrogen (N) and phosphorus (P) in the early stages of development of *Medicago sativa* seedlings, using RT-PCR, Western blotting and confocal microscopy. The response of NMH7 can be divided into two periods. The first period is 1-2 days post-germination, during which mRNA and protein expression are not affected by the presence or absence of these macronutrients in the media in any combination. In the second period (3-5 days post-germination), negative regulation of NMH7 expression was observed in plants treated with N plus P; in plants grown in media lacking both N and P, expression is constitutive. A synergic role of both nutrients in the regulation of NMH7 is proposed. NMH7 is localized in the cytoplasm during the first period, whereas in the second period it is localized mainly in the nuclei. By studying a developmental series of N-starved seed and seedlings, we provide the first evidence for differential sub-cellular localization of NMH7 during seedling establishment. As in seedlings, nitrate *per se* is not enough to inhibit NMH7 expression in nodules, but its cellular localization is affected. We discuss the implications of these results in the nutritional regulation of NMH7 and its role in nodule development.

Key Words: *Medicago sativa*, Nitrogen fixation, Phosphorous, NMH7, MADS domain transcription factor, Confocal microscopy, Nitrate.

INTRODUCTION

Dinitrogen (N_2) fixation is an important process in the mineral nutrition of plants (Aerts & Chapin 2000), and in controlling responses of ecosystems to global environmental change. Where nitrogen (N) is low in supply, N-fixers have an advantage: they can fix N_2 into biomass and thereby grow faster than competitors. In contrast, where N is abundant, the high energetic cost of N_2 fixation (Gutschick 1981) discriminates against N_2 fixers, which are competitively excluded by non-fixing species (Vitousek et al. 2002). Establishment of N fixing symbiosis between leguminous plants and soil bacteria of the family Rhizobiaceae has given rise to a new organ: the nodule. Symbiosis requires a constant fine-tuned signal exchange between plants and bacteria (Patriarca et al. 2004). Flavonoids excreted from roots of leguminous plants activate the synthesis of Nod factor to trigger nodule morphogenesis. In *Medicago sativa* plants, flavonoid production by roots increases under N limitation (Coronado et al. 1995). This increase is accompanied by enhanced activity of *Sinorhizobium meliloti* nod genes (Silveira et al. 1998). In certain genotypes of *M. sativa*, N starvation is sufficient to induce the development of empty (bacterium free) nodules (Truchet et al. 1989). In the soil, establishment of symbiosis is regulated by N (mainly nitrate, NO_3^-), but the initial development of nodules in the susceptible zone behind the root tip generates signals that limit subsequent rhizobia infection, a mechanism known as autoregulation (Limpens & Bisseling 2003).

Application of exogenous nitrogen inhibits all phases of nodulation, including bacterial infection, nodule development and N fixation (Carroll & Mathews 1990). High concentrations of NO_3^- promote a systemic inhibition of nodulation involving interaction with autoregulatory mechanisms (Forde & Lorenzo 2001). Hypernodulation mutants are

77 simultaneously tolerant to NO_3^- yet defective in their autoregulatory response (Duc &
78 Messager 1989).
79 Both theory (Wang, Houlton & Field 2007) and experimental evidence (van Groenigen et
80 al. 2006) indicate that symbiotic N fixation increases with increasing phosphorus (P)
81 availability; conversely, N_2 fixers can accelerate soil P availability. This interaction is
82 critical to successful nutrition and nodulation of N fixing plants. In legumes, P is often the
83 limiting nutrient for growth, and P deficiency can impair nodulation and N fixation (Tang
84 et al. 2001). Recent studies have shown that, at high N concentration, P has a counteracting
85 effect on the inhibitory action of N in the early stages of nodulation (Gentili & Huss-Danell
86 2003; Gentili, Wall & Huss-Danell 2006). Evolution has endowed plants with an array of
87 adaptative responses to low N and P, which are manifest at morphological, physiological,
88 and biochemical levels (Walch-Liu et al. 2006).
89 MADS domain transcription factors may be important regulators of specific development
90 processes that integrate the response to biotic and abiotic environments. *ANR1* is a key
91 regulator of lateral root growth in response to external NO_3^- in *Arabidopsis thaliana*, and
92 six other MADS-box genes respond to N in a similar way to *ANR1* (*AGL14*, *AGL16*,
93 *AGL19*, *SOC1* and *AGL21*). Expression of *SOC1* changes in response to the supply of P
94 and sulfur (S) (Gan et al. 2005).
95 There is striking similarity between lateral roots and undifferentiated nodules. Both plant
96 organs require auxins for primordial development and for vasculature differentiation. The
97 development of both structures can be regulated by the N and P status of the plant, and
98 pathway overlap has been proposed during early developmental stages (de Billy et al. 2001,
99 Ferguson, Roos & Reid 2005). *NMH7* is among several MADS box genes (with *NMHC5*
100 and *NGL9*) that are expressed exclusively in undetermined nodules of *M. sativa* and in

flowers (Zucchero, Caspi & Dunn 2001). However, we found that *NMH7* is also expressed in non-inoculated seeds and N-starved seedlings of *M. sativa*. The presence of NMH7 prior to bacterial colonization suggests that this protein is involved in non-symbiotic events or in the developmental program relating to bacterial colonization (Páez-Valencia et al. 2008a).

To gain further insight into regulatory functions of the MADS-domain protein NMH7 in seedlings and nodules, the present work had three aims: (1) to investigate the nutritional regulation of NMH7 by analyzing how N, P and their interaction affect the expression of NMH7 in *M. sativa* seedlings; (2) to determine whether the subcellular localization of NMH7 depends on the nutritional state of the plant; and (3) to analyze the relationship between NO_3^- inhibition of nodulation, and the expression and localization of NMH 7.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *Medicago sativa* were surface sterilized with 2.5% sodium hypochlorite and rinsed five times with distilled water. The seeds underwent imbibition for two hours in distilled water, and were aseptically transferred to moistened filter paper containing mineral solution (Rigaud & Puppo 1975) supplemented (or not) with N or with P, and balanced for cations and anions with NaCl. As potassium (K) interacts with N and P, we used sodium salts in our nutritional solutions (Tsai et al. 1993). The seeds underwent germination at 20^o C in the dark for 48 hours; germination was defined as radicule protrusion. After imbibition, the seeds were treated with four different nutrient solutions. Complete nutrient solution contained: 10 mM $NaNO_3$, 0.5 mM Na_2HPO_4, 0.25 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 43 μM sodium iron (Fe^{3+}) ethylene-diamine-tetra-acetic acid (EDTA), 0.16 μM $CuSO_4$ $5H_2O$, 0.38

μM $ZnSO_4$ $7H_2O$, 1.8 μM $MnSO_4$ H_2O, 45 μM H_3BO_3, and 0.014 μM Na_2MoO_4 $7H_2O$ (pH adjusted to 7.5). Na_2HPO_4 and NaH_2PO_4 were replaced by 0.96 mM NaCl in the solution lacking P. In the solution lacking N, $NaNO_3$ was replaced by 1.6 mM NaCl; meanwhile, in control P and N nutrients were substituted by 2.60 mM NaCl. The four treatments were (1) control (i.e., without N and P), (2) nitrogen added (+N), (3) phosphorus added (+P), and (4) nitrogen and phosphorus added (+NP). Plants were maintained in a growth chamber with day/night temperature 20/25^{o}C and a 14 hour photoperiod.

Plant N and P concentrations

Plants were harvested for nutrient analysis after 1, 2, 3, 4, 5, 6 and 7 days. Masses sampled from each individual were mixed and, from this stock, two sub-samples were drawn; both were used to analyze nutrient concentrations.

All subsamples for nutrient analysis were oven dried at 60 ºC for 48 hours and then ground (mesh size 40 μm). Nitrogen and P content were determined by Kjeldahl digestion; the samples were digested with 7 ml of concentrated H_2SO_4, 1.1 g of digestion mixture (K_2SO_4 and Cu_2SO_4, 9:1) and 3 ml of H_2O_2. Extracts and standards were analyzed colorimetrically using an Auto Analyzer II (Bayer, Germany).

Effect of nitrate on nodule development

For inoculation assays, seeds of *M. sativa* were surface sterilized with 2.5% sodium hypochlorite and rinsed five times with distilled water. The seeds underwent imbibition for two hours followed by germination for 48 hours at 25 ºC in the dark on moistened filter paper. The resulting two-day old seedlings were transferred to a glass tube (diameter 20

mm, length 200 mm) containing N-free mineral solution (Rigaud & Puppo 1975); the tube was stopped with cotton plugs and partly wrapped with opaque paper (Ligero et al. 1987). Ten-day-old seedlings were inoculated with *Sinorhizobium meliloti* strain 1021 (2×10^5 cells). The tubes were divided in two sets; in the first set (control) the plants were maintained in N-free solution and the nodules were harvested 35 days post inoculation (dpi). In the second set, 21 dpi plants grown in N free medium were transferred to a medium containing 18 mM $NaNO_3$, and the nodules were harvested seven days later (i.e., at 35 dpi).

Statistical analysis

Statistical analyses for nutrient concentration were performed using JMP 5.0.12; all statistical tests involved analysis of variance (ANOVA) by time and by treatment. Data were log-transformed where necessary. A significance level of $P = 0.05$ was chosen in all of these tests.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis

Total RNA from developing *M. sativa* seedlings was extracted by means of Trizol Reagents according to the manufacturer's protocol (Invitrogene). RNA was quantified spectrophotometrically, and RNA integrity was ensured by electrophoresis. RNA was treated with DNaseI (Invitrogene). cDNA was synthesized from 1µg of total RNA using the Super Script II RNase H Reverse Transcriptase system (Invitrogene). *Rhe2* constitutive expression was used as an RT-PCR control (Bauer et al. 1994). Primers for PCR were as

follows: for the NMH7 complete coding sequence: NMH7F 5' CCATGGCTCGAGGAAAGATC3' and NMH7 R 5' GGATCCGGTGAGATCCGATC3'; for the *Rhe2* Rhe2F 5'CAGCCCATGATCAGCTCCCC3', and Rhe2R GAACCTGCTAGGCCAAGC3'. All the experiments were performed in triplicate.

Western blotting analysis

Proteins isolated from *M. sativa* from different treatments at different stages of development were analyzed by Western blotting as described previously (Páez-Valencia et al. 2008a) with a rabbit anti NMH7 K-C domain specific antibody in 0.1% Tween 20, 10 mM Tris, 150 mM NaCl, pH 7.4 containing 5% non fat dried milk.

Histochemical analysis and microscopy

Seedlings and nodules were fixed in FAA (3.7% formaldehyde, 50% Ethanol, 5% Acetic Acid) for three hours at room temperature, dehydrated in an ethanol series (25, 50, 75, and 100%) and then infiltrated with t-butyl alcohol (Sigma USA) diluted in ethanol in a stepwise series (25, 50, 75, and 100%) at 60 ^{o}C. This was followed by infiltration with paraplast and several changes of pure molten paraplast at 60 ^{o}C. Slide sections 5-10 μm thick were used for histochemistry and indirect immunofluorescence. For histochemistry, the slides were deparaffinized in xylene, hydrated in a graded alcohol/water series, and stained with PAS (Periodic Acid Shift)/haematoxiline or Toluidine Blue as according to Harris, Spence & Oparka (1994). For indirect immunofluorescence, slides with tissue sections were deparaffinized with xylene and rehydrated through an ethanol/water series. After deparaffination and hydration the slides were washed in PBS. To recover antigenicity, histological sections were immersed in Antigen Retrieval Citra Plus solution (BioGenex

HK080-5K) and were autoclaved for five minutes. The slides were pre-treated with Power Universal Reagent (BioGenex HK080-5K) to reduce non-specific, binding and were incubated with primary antibody (rabbit NMH7 -KC) at dilution 1:200 in PBS Tween preimmune sera for one hour at room temperature. Three rinsings with PBS were followed by incubation with Texas Red-conjugated anti-rabbit secondary antibody or with FITC conjugated rabbit secondary antibody. After several washes, the coverslips were mounted on slides with Vecta-shield, with Evans blue or propidium iodide. These preparations were analyzed by confocal microscopy at room temperature.

RESULTS

Nutrient concentrations

Analysis of variance of N and P concentrations and the N:P ratio among the days found that time variation within each period (i.e., in the first two days, and days three to seven) was statistically indistinguishable (Table 1). Differences in N and P concentrations and N:P ratio between periods in the control and +NP addition plants were low and non-significant (Table 2). In contrast, N or P enrichment significantly increased plant N and P concentrations with time ($F = 10.75$ for N, and $F = 6.368$, for P; $P < 0.01$).

Addition of N plus P consistently increased plant N and P concentrations in both study periods, relative to controls (by 52% in the first two days and by 45% in days three to seven for N, and by 67% and 27% for the first and second period respectively in the case of P); see Tables 1 & 2. Application of N or P alone did not, however, affect plant concentrations of N and P in the first period (i.e., first two days). In contrast, in the second period (days 3-7) the addition of N alone significantly increased N and P concentrations, by about 64%

and 67% respectively, relative to controls. Ratios of N:P were consistent across treatments within each of study period.

Effects of N and P on the expression of NMH7 (both mRNA and protein) in non inoculated seedlings of *M. sativa*

We investigated the effect of N and P on the expression of NMH7. *NMH7* was present in the four treatments during the first and second days. However, in plants treated with N and P simultaneously, a significant decrease on *NMH7* expression was detected from the third day. In control plants (both N and P-starved) the expression of *NMH7* was constitutive during the five days. No *NMH7* transcript was detected during the third and fourth day in the +N and +P treated plants (Fig. 1A). NMH7 polypeptide was detected uniformly in the +N, +P and control plants during the five days; NMH7 was not detected from the third day in plants treated with N plus P (Fig 1B).

Subcellular localization of NMH7 in seeds and N-starved seedlings

The NMH7 subcellular distribution was determined in a developmental series of *M. sativa* seeds and N-starved seedlings. *Seeds*: NMH7 is localized specifically in the cytoplasm of parenchymal cells from cotyledons or from embryonary root showing a punctuate pattern. No signal was detected in the nuclei (Fig 2A). *Seedlings 1 day old*: A cytoplasmic signal of NMH7 was detected in cotyledon cells (Fig 2B). No signal was detected in the nuclei (Fig2B3). *Seedling 2 days old:* NMH7 remains exclusively in the cytoplasm of the root cap, cortex parenchyma cells and lateral cofia cells of primary roots (Fig 2C). No signal is detected in the nuclei of the root cap cells (Fig 2C3). NMH7 is localized in the cytoplasm of root parenchyma cells and non signal is detected in the vascular tissues as it was

described (Fig2C4-5) *Seedlings of 3-4 days*: At these stages of development, NMH7 is localized mainly in the nuclei of cortical cells in N-starved seedlings (Fig. 2D). An amplification shows the specific nuclear localization of NMH7 (Fig. 2D2-4)

Effects of nitrate on nodule development and NMH7 expression

In our system, NO_3^- inhibits nodulation meanwhile NMH7 expression is maintained at mRNA and protein levels (Fig. 3I and 3II). An effect of N on nodule development was observed. The nodules were small and spherical, with no visible differentiation of the central tissues into zones; this is in contrast with nodules of control plants, which were rod shaped with the zonation typical of undetermined nodules (Zone I meristematic zone, Zone II invasion zone, Zone III fixation zone, and Zone IV senescence zone); see Fig. 3IA. In the nodules of NO_3^- treated plants, growth is arrested and the nodule endodermis surrounds the apical meristem (Fig. 3IB). These nodules display evidence of senescence, and there is also bacterial degradation. Bacteria maintain their peripherical structure, with a loss of cell content (Fig. 3IB4 and 6 arrow). These morphological alterations correspond to type I senescent cells. Type II senescent cells are also observed, where cell wall breakage is evidence of cell death (Fig. 3IB4 and 6 arrowheads).

To determine whether nitrate also affects carbohydrate in our system, as described previously (Matamoros et al. 1999), starch granules were analyzed in the nodules treated with NO_3^-. We found a significant reduction in their number relative to controls (Fig. 3IA4-5 and Fig 3IB6).

We also analyzed the effect of NO_3^- on nodule expression of NMH7 at both mRNA and protein level (Fig. 3II). NMH7 was detected in both control and NO_3^--treated plants, indicating that NO_3^- alone, as in seedlings, does not regulate the expression of this gene.

NMH7 is localized in uninfected cells of nitrate treated nodules

NMH7 mRNA and protein were detected in NO_3^- treated nodules. We therefore analyzed the localization of NMH7 by indirect immunofluorescence and confocal microscopy. NMH7 is detected only in uninfected cells of NO_3^- treated nodules (Fig 4). The localization of NMH7 is correlated with the structural integrity of the uninfected cells (Fig. 4B). Infected cells show clear evidence of senescence, accompanied by nuclear morphological changes including chromatin clumping into patches and decrease in nuclear diameter (Fig. 4C; Yamada et al. 2006). The uninfected cells, where NMH7 is localized, maintain normal nuclear morphology (Fig. 4D1-3).

DISCUSSION

Seedling establishment and competitiveness in an infertile environment are linked with the ability to adjust to nutrient stress and take advantage of nutrients as they become patchily available (Fitter 1994). In *M. sativa*, for NMH7 both mRNA and protein are regulated synergically by N and P; expression of NMH7 is stimulated in plants grown with limited N and P. The presence of both nutrients in the medium totally inhibits NMH7 expression, however (Fig. 1A and 1B). The protein and mRNA accumulation patterns both follow a similar trend. There is clear presence of the transcription factor in the four treatments

289 during the first two days after imbibition, but no NMH7 expression was detected in 3-5 day
290 old seedlings grown under optimal N and P nutritional conditions (Tables 1 and 2). These
291 results suggest that there are two clear developmental stages of *Medicago* seedling. The
292 first period reflects the physiological state of the plant, in which the endogenous N and P
293 concentrations are unaltered by the nutritional conditions, and in the second period they
294 change in a manner consistent with the expression pattern (Table 2). NMH7 activity can
295 also be regulated by its subcellular localization. Nuclear localization of the protein was
296 observed on the third day in plants growing under nutrient starved conditions. The changes
297 in the endogenous N and P endogenous concentration, in NMH7 expression and in
298 subcellular NMH7 localization suggest that NMH7 is part of the nutrient sensing
299 machinery that activates or suppresses the nodulation program in response to nutritional
300 cues. Our results suggest that there is a developmental switch occurring at the second
301 period, to prepare the seedling to cope with the environmental conditions. A role of MADS
302 box genes in the general response to nutrient stress has been suggested previously (Gan et
303 al. 2004).
304 Cross-talk between the signaling pathways for N or P deprivation has been suggested in
305 other studies. The deprivation of a single nutrient induces a range of ion transporters, such
306 as aquaporins, and phosphate transporters are up-regulated by nitrate (Wang, Garvin &
307 Kochian 2002). Transcription of MYB-like genes responds to N and P deprivation in
308 *Arabidopsis thaliana* (Todd et al. 2004). Our results also show the importance of studying
309 the effect of more than one nutrient at a time, as suggested by Hellsten & Huss-Danell
310 (2000) and Tsai et al. (1993).
311 Our observations with N-starved seed and seedlings revealed a previously unknown
312 developmentally regulated NMH7 protein localization in the nucleus and cytoplasm (Fig.

313 2). Regulation of gene expression by extracellular signals often requires that transcription
314 factors for effectors remain inactive until an external signal promotes their activation. This
315 is achieved in many signaling pathways through the sequestration of inducible transcription
316 factors in the cytoplasm (Zeigler & Ghosh 2005). The response to nutrient starvation could
317 be regulated by control of the subcellular localization of a transcription factor (Beck & Hall
318 1999). NMH7 is localized in the cytoplasm of seeds and first period seedlings. During the
319 second period, it is localized in the nucleus of cortical cells when the N level is related to
320 environmental conditions. These results suggest that NMH7 nucleus localization is a
321 response to nutrient. However, the question of whether N limitation in the
322 legume/Rhizobium spp. symbiosis simply represents a nutritional deficiency, or is also a
323 signal that is locally perceived by roots that then leads to nodulation is still open. Our data
324 collectively suggest that nuclear localization of NMH7 is developmentally regulated by the
325 N and metabolic status of the plants. The developmental regulation of MADS domain
326 transcription factors in subcellular localization is well documented. For example, AGL 15
327 (AGAMOUS like-15) is localized in the cytoplasm of cells of the female germ unit before
328 fertilization, and moves into the nucleus of the few dividing cells in the embryo (Perry,
329 Lehti & Fernández 1996; Perry, Nichols & Fernández 1999). We have shown that NMH7
330 can interact with a Fructose 1-6 bisphosphate aldolase either in cytoplasm or the nucleus in
331 N-starved seedlings. It is therefore plausible that, in *M. sativa,* aldolase-NMH7 relates the
332 metabolic status with the central developmental network (Páez-Valencia et al. 2008b).
333 APETALA 3 (AP3) is a putative orthologue of NMH7 in *Arabidopsis*. AP3 or
334 PISTILLATA (PI) are localized in the cytoplasm, but they interact to form AP3/PI
335 heterodimers, promoting their translocation to the nucleus (McGonigle, Bouhidel & Irish

336 1996). Similarly, the Petunia SEPALLATA-like protein FBP11 interacts with FBP2, FBP5
337 or FBP9a so as to be translocated to the nucleus (Immink et al. 2002).
338 An alternative mechanism for nuclear translocation is phosphorylation. The translocation
339 from the cytoplasm to the nucleus of AGL24, a MADS domain protein, implies its
340 phosphorylation by MRLK (meristem receptor like kinase, Fujita et al. 2003). NMH7 is a
341 phosphoprotein, and its phosphorylation state may change during development (data not
342 shown). Our current research aims to correlate the phosphorylation/dephosphorylation
343 status of NMH7 with its intracellular localization and transcriptional activity.
344 NO_3^- inhibition of undetermined nodules of *M. sativa* was observed (Fig. 3). In NO_3^- -
345 treated nodules, NMH7 is exclusively localized in uninfected cells, in contrast with normal
346 nodules (Fig 4), whereas NMH7 protein is localized in infected cells, particularly in the
347 invasion zone (zone II), interzone (II-III) and N fixing zone (IV); no signal was detected in
348 uninfected cells (Páez-Valencia et al. 2008a). Re-localization of NMH7 in uninfected cells
349 could indicate the intercellular transit of the protein through plasmodesmata, since
350 secondary plasmodemata form in response to changes in the developmental program
351 (Crawford & Zambriski 2001).
352 Nitrate is a transient or seasonal stress in many soils. If NO_3^- mineralization is not followed
353 quickly by substantial rains, that leach N from the rooting zones of germinating annual
354 legumes, then NO_3^- can significantly disrupt nodulation in susceptible species (Harper &
355 Gibson 1984). It is likely that some symbiosis can delay nodulation in the presence of soil
356 NO_3^-, and then nodulation proceeds when soil reserves of N are diminished. NMH7
357 localized in the cytoplasm of uninfected cells could be a signal that puts on standby the
358 nodule development program, since the inhibitory effects of NO_3^- are reversible. This
359 developmental plasticity could be an adaptive strategy to buffer NO_3^- -induced stress in

360 relation to nodulation. Our results suggest that uninfected cells act as a reservoir of cellular
361 components involved in the reactivation of nodule development, protecting them against
362 oxidative damage induced by NO_3^-. This has been found for ferritine, the antioxidant
363 involved in the sequestration of catalytic ions, which is localized mainly in uninfected cells
364 after two days of NO_3^- treatment (Matamoros et al. 1999). NMH7 could therefore be
365 involved in regulating the restarting of the nodule developmental program. It is important to
366 determine in future whether there is a correlation between the temporal window
367 reversibility of nodule morphogenesis treated with nitrate, and NMH7 expression. Different
368 models have been proposed to define the threshold after which senescence becomes an
369 inevitable consequence of exposure to stress. For example, the decay model suggests that
370 there is a point of no return when the breakdown in the endogenous defense processes does
371 not control oxidation and leads to senescence (Leshem 1988; Swaraj & Bishnoi 1996).
372 Induction of changes in nodule development by nutrient stress could be an excellent way to
373 study the cellular behavior of regulatory proteins when classical genetic and/or
374 biotechnological tools are inadequate. Nitrate-treated nodules produce aberrant
375 morphologies that resemble those produced by transgenic plants upon silencing by RNAi
376 the expression of key components of nodule morphogenesis. For example, analysis of
377 *MtENOD40-1and MtENDO40-2 RNAi* nodules showed that zone differentiation of central
378 tissue could not be seen; the majority of these nodules were senescent, affecting also
379 bacteroid development (Wan et al. 2007). Similarly, nodules treated with NO_3^- produced
380 senescence in plant tissues and bacteroids (data not shown); expression of NMH7 is not
381 detected, however, in infected tissues, suggesting an alternative role in nodule development
382 in addition to infected cell differentiation, as proposed by Heard & Dunn (1995).
383

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REFERENCES

Aerts R. & Chapin F.S. (2000) The mineral nutrition of wild plants revisited: a re-evaluation of processes and patterns. *Advances in Ecological Research* **30**, 2-67.

Bauer P., Crespi M., Szecsi J., Allison L., Shultze M., Ratet P., Kondorosi E. & Kondorosi A. (1994) Alfalfa *ENOD12* genes are differentially regulated during nodule development by nod factor and *Rhizobium* invasion. *Plant Physiology* **105**, 585-592.

Beck T. & Hall M. (1999) The TOR signaling pathway controls nuclear localization of nutrient regulated transcription factor. *Nature* **402**,689-692.

Carroll B.J. & Mathews A. (1990) Nitrate inhibition of nodulation legumes. In *Molecular Biology of Nitrogen Fixation* (ed. Gresshoff P.M.), pp159-180. CRC Press, Boca-Raton Florida.

Coronado C., Zuanazzi J., Sallaud C., Quirion J., Esnault R., Husson H., Kondorosi A. & Ratet P. (1995) Alfalfa root flavonoid production is nitrogen regulated. *Plant Physiology* **108**, 533-542.

Crawford K. & Zambryski P. (2001) Non-targeted and targeted protein movement through plasmodesmata in leaves in different developmental and physiological states. *Plant Physiology* **125**, 1802-1812.

de Billy F., Grosjean C., May S., Bennett M. & Cullimore J. (2001) Expression studies on AUX1-like genes in *Medicago truncatula* suggest that auxin is required at two steps in early nodule development. *Molecular Plant Microbe Interaction* **14**,267-277.

Duc G. & Messager A. (1989) Mutagenesis of pea (*Pisum sativum* L.) and the isolation of mutants for nodulation and nitrogen fixation. *Plant Science* **60**, 207-213.

Ferguson B., Ross J. & Reid J. (2005) Nodulation phenotypes of gibberellin and brassinosteroid mutants of pea. *Plant Physiology*, **138**, 2396-2405.

Fitter A.H. (1994) Architecture and biomass allocation as components of the plastic response of root systems to soil heterogeneity. In *Exploitation of Environmental Heterogeneity by Plants.* (eds M.M. Caldwell & R.W. Pearcy), pp. 305-323. Academic Publishers, San Diego.

Forde B. & Lorenzo H. (2001) The nutritional control of root development. *Plant and Soil* **232**, 51-68.

Fujita H., Tekemura M., Tani E., Nemoto K., Yolota A. & Kohchi T. (2003) An *Arabidopsis* MADS-box protein, AGL24, is specifically bound to and phosphorylated by meristematic receptor-like kinase (MRLK). *Plant & Cell Physiology* **44**, 735-742.

Gan Y., Filleur S., Rahman A., Gotensparre S. & Forde B. (2005) Nutritional regulation of *ANR1* and the root-expressed MADS-box genes in *Arabidopsis thaliana. Planta* **222**, 730-742.

Gentili F. & Huss-Danell K. (2003) Local and systemic effects of nitrogen and phosphorus on nodulation and nodule function. *Journal of Experimental Botany* **54**, 2757-2767.

Gentili F., Wall L.G. & Huss-Danell K (2006) Effects of phosphorus and nitrogen on nodulation are seen already at the stages of early cortical cell division in *Alnus incana*. *Annals of Botany* **98**, 309-315.

Gutschick V. (1981) Evolved strategies in nitrogen acquisition by plants. *The American Naturalist* **118**, 607-637.

Harper J. & Gibson A. (1984) Differential nodulation tolerance to nitrate among legumes species. *Crop Science* **24**, 797-801.

Harris N. Spence J. & Oparka K.J. (1994) General enzyme histochemistry. In *Plant Cell Biology: A Practical Approach* (ed. Harris N. & Oparka K.J.) pp 51-68. IRL Press, Great Britain.

Hellsten A and Huss-Danell K. 2000. Interaction effects of nitrogen and phosphorus on nodulation in red clover (Trifolium pratense L.). Acta Agriculturae Scandinavica B 50: 135-142.

Heard J. & Dunn K. (1995) Symbiotic induction of a MADS-box gene during development of alfalfa root nodules. *Proceedings of the National Academy of Sciences USA* **92**, 5273-5277.

Immink R., Gadella T., Ferrario S., Busscher M. & Angenet G. (2002) Analysis of MADS box protein-protein interactions in living plant cells. *Proceedings of the National Academy of Sciences USA* **99**, 2416-2421.

Leshem Y. (1988) Plant senescence processes and free radicals. *Free Radicals Biology and Medicine* **8**, 39-49

Ligero F., Lluch C. & Olivares J. (1987) Evolution of ethylene from roots and nodulation of alfalfa (*Medicago sativa* L) plants inoculated with *Rhizobium meliloti* as affected by presence of nitrate. *Journal of Plant Physiology* **129**, 461-465.

Limpens E. & Bisseling T. (2003) Signaling in symbiosis. *Current Opinnion in Plant Biology* **6**,343-350.

455 Matamoros M., Baird L., Escuredo P., Dalton D., Minchin F., Iturbe-Ormaetxe I., Rubio
456 M., Moran J., Gordon A. & Becana A. (1999) Stress-induced legume root senescence.
457 Physiological, Biochemical and structural alterations. *Plant Physiology* **121**, 97-112.
458 McGonigle B., Bouhidel K. & Irish V. (1996) Nuclear localization of the Arabidopsis
459 APETALA3 and PISTILLATA homeotic gene products depends on their simultaneous
460 expression. *Genes and Development* **10**, 1812-1821.
461 Páez-Valencia J., Sánchez-Gómez C., Valencia-Mayoral P., Contreras-Ramos A.,
462 Hernández-Lucas I., Orozco-Segovia A. & Gamboa-deBuen A. (2008a) Localization of the
463 MADS domain transcription factor NMH7 during seed, seedling and nodule development
464 of *Medicago sativa. Plant Science* **175**, 596-603.
465 Páez-Valencia J., Valencia-Mayoral P., Sánchez-Gómez C., Contreras-Ramos A.,
466 Hernández-Lucas I., Martínez-Barajas E. & Gamboa-deBuen A. (2008b) Identification of
467 Fructose-1,6 bisphophate aldolase cytostolic class I as an NMH7 MADS domain associated
468 proteína. *Biochemical and Biophysical Research Communications* 376; 700-705
469 Patriarca E., Tate R., Ferraioli S. & Iaccarino M. (2004) Organogenesis of legume nodules.
470 *International Review of Cytology* **234**, 201-262.
471 Perry S., Lehti M. & Fernández D. (1999) The MADS-domain protein AGAMOUS-like 15
472 accumulates in embryonic tissues with diverse origins. *Plant Physiology* **120**, 121-130.
473 Perry S., Nichols K. & Fernández D. (1996) The MADS-domain protein AGL15 localizes
474 to the nucleus during early stages of seed development. *Plant Cell* **8**,1977-1989.
475 Rigaud J. & Puppo A. (1975) Indole-3-acetic acid catabolism by soybean bacteroids.
476 *Journal of General Microbiology* **88**, 223-228.

Silveira J., Clergeot P., Quirion J., Husson H., Kondorosi A. & Ratet P. (1998) Production of *Sinorhzobium meliloti nod* gene activator and repressor Flavonoids from *Medicago sativa* roots. *Molecular Plant Microbe Interactions* **11**,784-794.

Swaraj K. & Bishnoi N. (1996) Physiological and biochemical basis of nodule senescence in legumes: a review. *Plant Physiology and Biochemistry* **23**, 105-116.

Tang C., Hisinger P., Drevon J.J. & Jaillard B. (2001) Phosphorous deficiency impairs early nodule functioning and enhances proton release in root of *Medicago sativa* L. *Annals of Botany* **88**,131-138.

Todd C., Zeng P., Rodriguez A., Hoyos M. & Polacco J. (2004) Transcripts of MYB-like genes respond to phosphorous and nitrogen deprivation in *Arabidopsis*. *Planta* **219**, 1003-1009.

Truchet G., Barker D., Camut S., de Billy F., Vasse J. & Huguet T. (1989) Alfalfa nodulation in the absence of *Rhizobium*. *Molecular and General Genetics* **219**, 65-68.

Tsai S., Bonetti R., Agbala S. & Rossetto R. (1993) Minimizing the effect of mineral nitrogen on biological fixation in common bean by increasing nutrient level. *Plant and Soil* **152**, 131-138.

van Groenigen K., Six J., Hungate B., de Graaff M. & van Breemen N. (2006) Element interactions limit soil carbon storage. *Proceedings of the National Academy of Sciences of the USA* **103**, 6571-6574.

Vitousek P., Cassman K., Clevaland C., Crews T., Field C., Grimm N.B., Howarth R., Marino R., Martinelli L., Rastetter E. & Sprent J. (2002) Towards and ecological understanding of biological nitrogen fixation. *Biogeochemistry* **57/58**, 1-45.

Walch-Liu P., Ivanov I.I., Filleur S., Gan Y., Remans T. & Forde B. (2006) Nitrogen regulation of root branching. *Annals of Botany* **97**, 875-881.

Wan X., Hontelez J., Lillo A., Guarnerio C., van de Peut D., Fedorova E., Bisseling T. & Franssen H. (2007) *Medicago truncatula ENOD40-1* and *ENOD40-2* are both involved in nodule initiation and bacteroid development. *Journal of Experimental Botany* **58**, 2033-2041.

Wang Y., Garvin D. & Kochian L. (2002) Rapid induction of regulatory and transporter genes in response to phosphorus, potassium and iron deficiencies in tomato roots. Evidences of cross talk and root/rhizosphere mediated signals. *Plant Physiology* **130**, 1361-1370.

Wang Y.P., Houlton B.Z. & Field C.B. (2007) A model of biogeochemical cycles of carbon, nitrogen, and phosphorus including symbiotic nitrogen fixation and phosphatase production. *Global Biogeochemical Cycles* **21**, GB1018.

Zeigler E. & Ghosh S. (2005) Regulation of inducible transcription factor through controlled localization. *Science STKE*, 2005, re6.

Zucchero J., Caspi J. & Dunn K. (2001) ngl9: a third MADS box gene expressed in alfalfa root nodules. *Molecular Plant Microbe Interaction* **14**, 1463-1467.

Table 1. Effects of nutrient addition and time on N and P concentrations in each study period.

Period	Effect	*F*	*P*
Days 1-2			
N	Treatment	10.47	**0.004**
	Time	0.707	0.410
	Treatment × Time	0.320	0.578
P	Treatment	8.45	**0.009**
	Time	0.484	0.495
	Treatment × Time	0.344	0.564
N:P ratio	Treatment	0.233	0.634
	Time	0.004	0.948
	Treatment × Time	0.009	0.925
Days 3-7			
N	Treatment	13.75	<**0.001**
	Time	0.006	0.936
	Treatment × Time	0.306	0.757
P	Treatment	1.770	0.189
	Time	0.186	0.668
	Treatment × Time	0.936	0.338
N:P ratio	Treatment	0.789	0.378
	Time	0.007	0.931
	Treatment × Time	0.349	0.557

Note: Significant results at an alpha level of 0.05 are shown in boldface.

Table 2. Nitrogen and P concentrations under four treatments of nutrient additions. Values are means ± SE.

Period	Control	+N	+P	+NP
Days 1-2				
N (mg g^{-1})	19.6 ± 2.0	12.5 ± 1.0	9.5 ± 0.5	12.9 ± 1.0
P (mg g^{-1})	2.0 ± 0.23	1.0 ± 0.07	1.0 ± 0.17	1.2 ± 0.16
N:P ratio	10 ± 1.2	12 ± 1.0	10 ± 1.3	12 ± 1.1
Days 3-7				
N (mg g^{-1})	22.4 ± 2.9	**25.2 ± 3.7**	**16.5 ± 1.9**	15.4 ± 1.0
P (mg g^{-1})	1.9 ± 0.14	**2.5 ± 0.32**	1.5 ± 0.31	1.5 ± 0.29
N:P ratio	12 ± 1.0	10 ± 1.2	12 ± 1.3	12 ± 1.9

Note: Significant differences between periods at an alpha level of 0.05 are shown in boldface.

FIGURE LEGENDS

Figure 1:

Effect of nitrogen and phosphorus on NMH7 expression.

A) Independent and combined effect of N and P on expression of the *NMH7* MADS-box gene at different stages of seedling establishment (1-5 days post-germination). Total mRNA was extracted from seedlings grown under different nutritional conditions (NP, N, P, control). RT-PCR was performed in triplicate for each treatment. *Rhe 2* primers were included in each reaction as control.

B) Effect of nitrogen and phosphorus on NMH7 protein. Seedlings at different stages of development under different nutritional conditions were collected (1-5 day post-germination), and equal amounts of protein (25μg) were transfer and developed with NMH7 specific antibodies. Western blotting was performed in triplicate for each treatment. All assays gave the same results.

Figure 2. Immunolocalization of NMH7 at early stages of *Medicago sativa* development. Tissues at different stages of *M. sativa* development were processed for indirect immunoflorescence using sera specific for NMH7 detected with goat anti rabbit coupled to FITC (green channel), and counterstained with Evans blue or propidium iodide (red channel). Evans blue marks the membrane and cytoplasm, and propidium iodide outlines all nuclei.

(A) Seeds parenchyma cells of embryonary cotyledon. (A1) stained with Evans blue (red channel). (A2) immunolocalization of NMH7 (green channel). (A3) merged image.

(B) Seedlings one day old (post germination). (B1-2) Magnification of parenchyma cells of cotyledon. Merge between NMH7 (green channel) and Evans blue stained structures (red channel). Parenchyma cells of cotyledon stained with propidium iodide show the cytoplasmic localization of NMH7 with a punctuate pattern. (B3). Nuclei (red) are indicated by arrows; no signal is detected

(C) Seedlings two day old (post germination) (C1-2) Cortical parenchyma cells and root cap cells of the primary root. NMH7 is present in an intense cytoplasmic localization (green channel); no signal is detected in nuclei stained with propodium iodide (red channel). (C3) Enlarged images of root cap cells show cytoplasmic localization of NMH7. (C4-5) Cytoplasmic localization of NMH7 parenchyma cortical cells; no signal is detected in vascular bundles.

(D) Seedlings three day old. (D1) Cortical cells of primary roots. Nuclear localization of NMH7 (nuclei are indicated by arrows). (D2-4) Magnification of cortical cells. (D2) The nuclei are contrasted by propidium iodide (red channel). (D3) NMH7 nuclear localization (green chanel). (D4) merged images. White boxes indicate enlargement. Abbreviations: sc: seed cotyledon, pc: parenchyma cells, cp: cortex parenchyma, co: cortical cells of primary root, rc: root cap. . Bar size: 50, 20 and 10μm.

Figure 3. (I) Histological comparison of control nodules (A) and NO_3^--treated nodules (B)

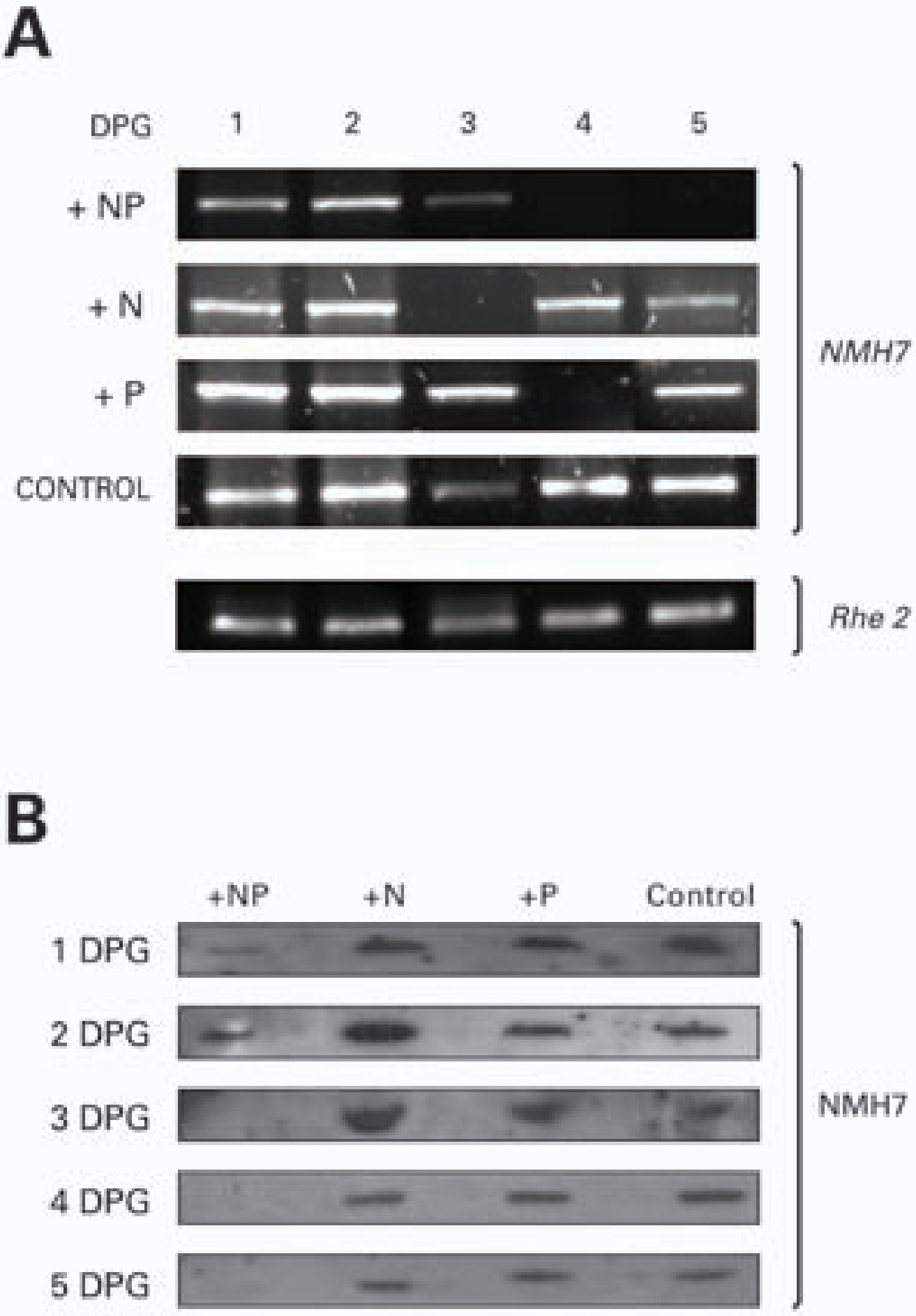
Light microscopy and histochemical analysis for carbohydrates (starch) with PAS reactive. (A1) Median longitudinal section of 5 week-old nodules. (A2) Magnification of the meristematic zone (I). (A3) Magnification of the prefixing zone (II) and interzone

II-III. (A4) interzone II-III with high amount of amiloplastes (A5) senescent zone (Zone IV).

(B1-B3) Optical microscopy analysis of a longitudinal section of NO_3^-- treated nodules; there is no zone differentiation. (B4-B6) Release of rhizobia from intracellular colonies (arrows) and senescent cells with loss of turgor and collapse (arrowheads); uninfected cells show few amilopastes. Abbreviations: sc: senescent cells, uc: uninfected cells, am: amiloplastes.

(II) NMH7, both mRNA and protein, are detected in NO_3^--treated and control nodules. (A) RT-PCR analysis (B) Western blotting analysis.

Figure 4. Subcellular localization of NMH7 in NO_3^--treated nodules. (A) Tissue sections were stained with toluidine blue to show the general morphology of nodules; no zonation can be seen. (B) Magnification of an uninfected cell; nuclear morphology is conserved. (C) Magnification of infected cells; nuclear alteration is related to cell senescence. (D1). Contrast phase of nodule cells. Asterisks indicate uninfected cells (D2) Localization of NMH7 in uninfected cells (red signal). (D3) merged image. Abbreviations: uc: uninfected cells, ic= infected cells, bar: 20μm.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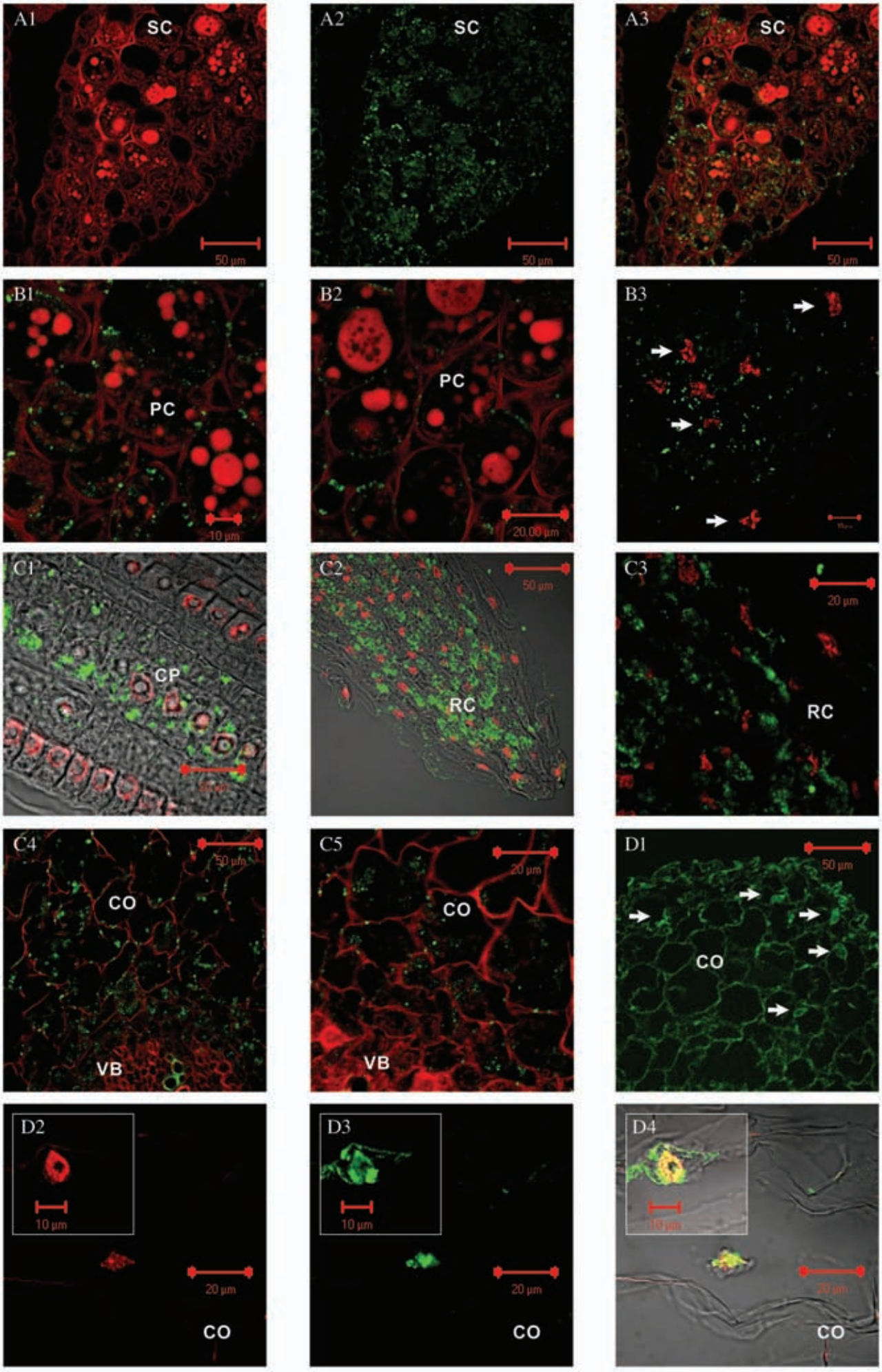
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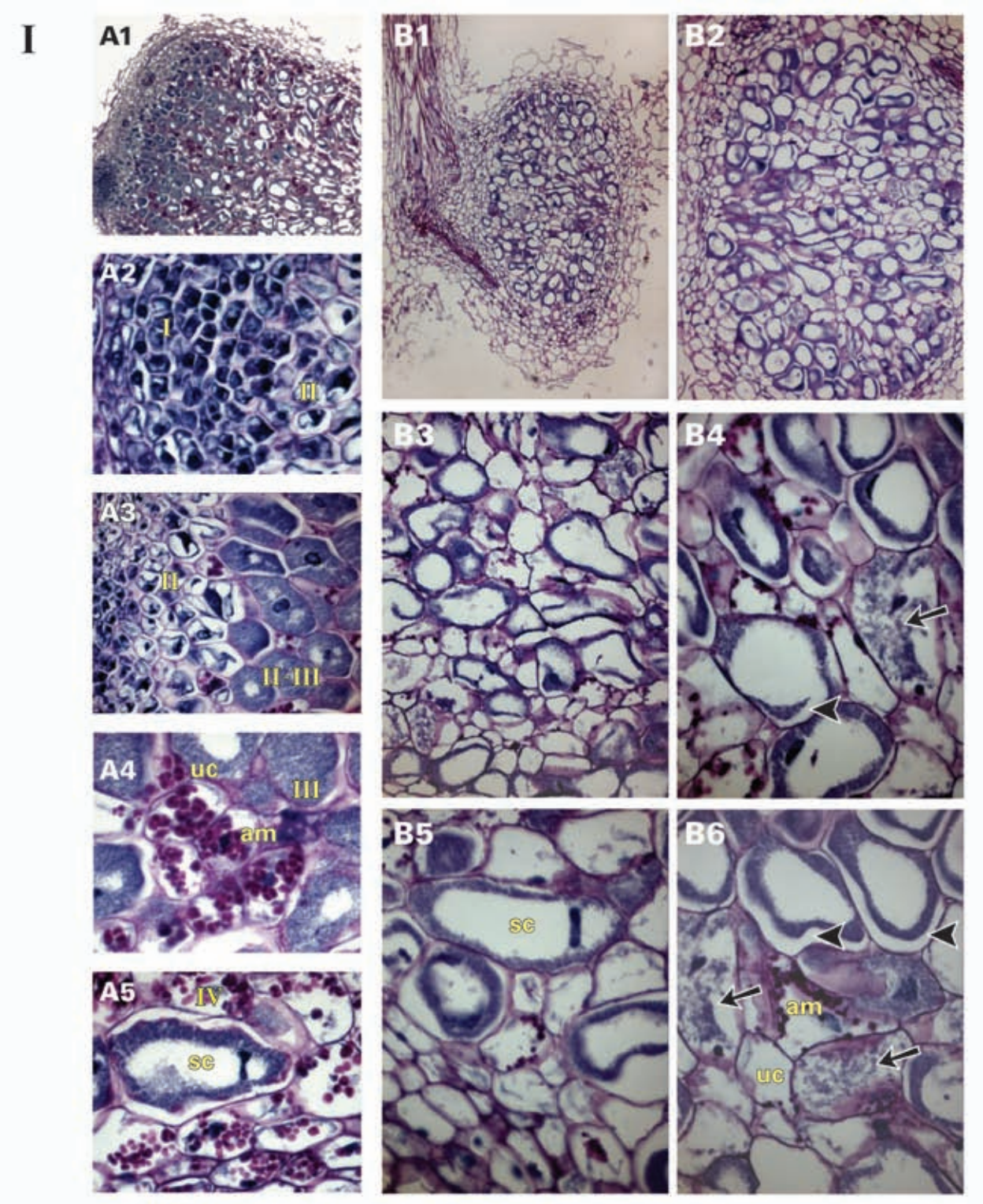
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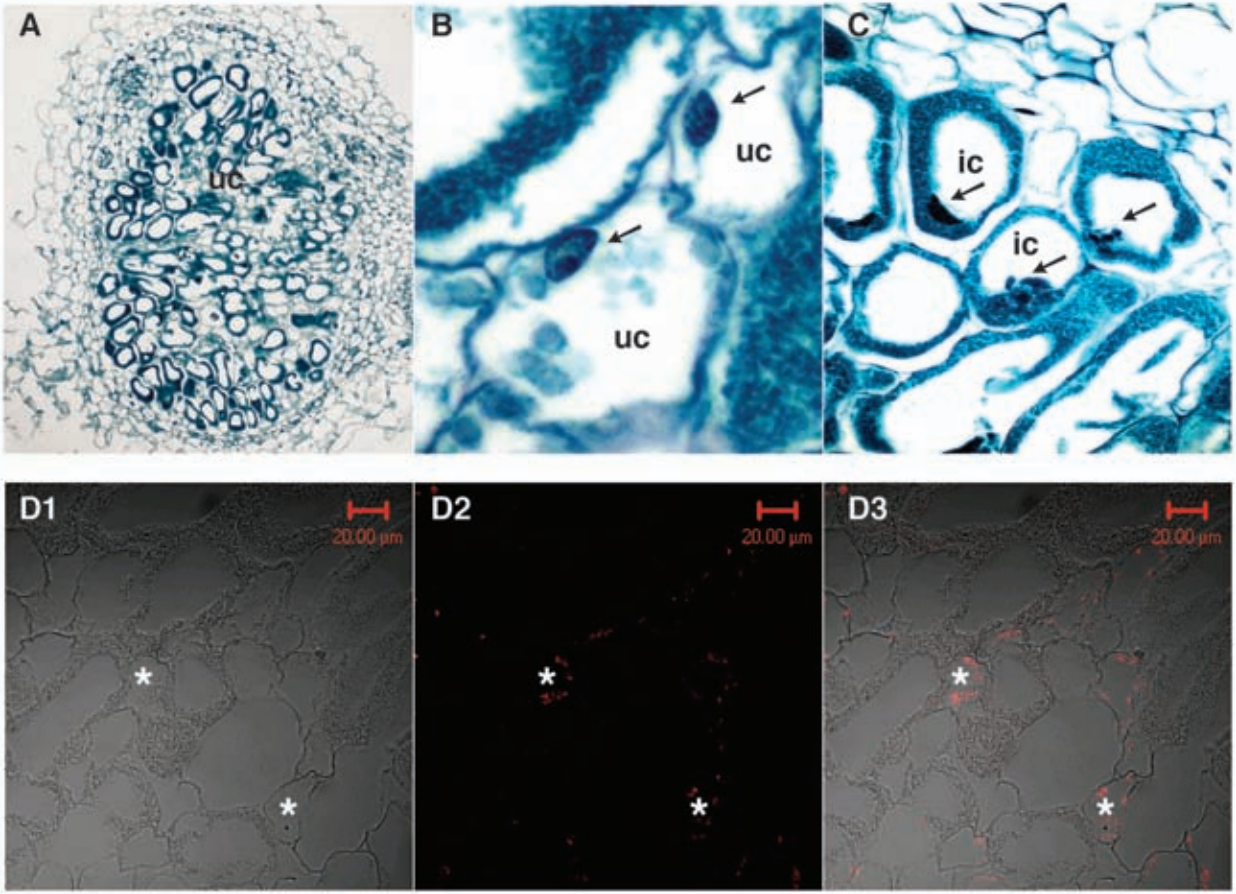
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Discusión

Perspectivas

Los productos hidrolizados de la sacarosa son usados para la biosíntesis de almidón y celulosa o son metabolizados por enzimas glicolíticas para producir fosfoenolpiruvato (PEP), el cual puede ser carboxilado a oxaloacetato y después ser reducido a malato para abastecer al bacteroide. Los estudios metabolómicos recientes han confirmado un incremento en la concentración de intermediarios glicolíticos y la síntesis de ácidos orgánicos en los nódulos de *Medicago sativa,* comparado con las raíces (Barsch et al. 2006). Además, la fructosa-6-fosfato y la glucosa-6-fosfato son 5 veces más abundantes en nódulos, mientras que las concentraciones de glucosa y fructosa son mucho menores (Desbrosses et al. 2005). La inducción y regulación de la fructosa-1,6-bisfofato aldolasa citosólica está asociada con la activación de la glicólisis durante el desarrollo del nódulo (Yahyaoui et al. 2004) y concomitantemente ocurre un aumento en la expresión de GS, asparargina sintasa (AS), aminotransferasas y nitrito reductasa mostrando que los metabolismos del N y del carbono (C) están integrados durante la simbiosis (Tesfaye et al. 2006). Muchas proteínas *moonlighting* alternan su función entre las rutas de transducción de señales y las interacciones con ácidos nucléicos; por ejemplo, la ferrodoxina-sulfito reductasa (Fd-EiR) se identificó a partir de preparaciones de nucleoide de los cloroplastos y se demostró que actuaba en la compactación del ADN, además de tener actividad de sulfito reductasa (Sato 2001). Por lo tanto, la Fs-SiR podría proteger directamente el ADN del cloroplasto minimizando la acumulación local del ión bisulfito y también podría actuar como un sensor del estado rédox ayudando a coordinar la expresión génica del plastoma con las condiciones metabólicas (Chi-Ham 2002). El papel central de la aldolasa regulando el metabolismo nitrogenado de las leguminosas antes y durante la simbiosis, perfila su interacción con NMH7 como enlace entre el estado metabólico y las redes centrales del desarrollo.

En el presente trabajo hemos demostrado que la adolasa interacciona con NMH7 y ambas proteínas son colocalizadas en el núcleo de células corticales de la raíz y de la capa interna de córtex del nódulo. El establecimiento del estadio de desarrollo y la condición nutricional que inducen la translocación nuclear de ambas proteínas (plántulas de 3-5 días no inoculadas y crecidas en deficiencia de N y P) nos permitirían analizar si el complejo Aldolasa-NMH7 se une a la región promotora de genes blanco regulando su expresión. Así mismo, la identificación de dichos genes nos ayudaría a identificar las redes de regulación genética dependientes de enzimas metabólicas que actúan como reguladores.

La aldolasa es una proteína que también desempeña funciones estructurales; se sabe que la interacción de la aldolasa con la actina puede estar modulada por los sustratos de la aldolasa y sus productos. Por lo tanto, otra línea de investigación podría ser el análisis de los efectos alostéricos de moléculas como la fructosa 1-fosfato; dihidroxicetona; fructosa-1,6-bisfofato y gliceraldehído 3 fosfato en la interacción entre NMH7 y la aldolasa *in vitro*. De esta manera, se podría establecer si una molécula indicadora del estado metabólico de la célula modula conformacionalmente a la aldolasa afectando su interacción con NMH7. Esta línea de investigación podría ayudarnos a entender cómo una señal metabólica se traduce en un evento de desarrollo que, en este caso, sería la interacción de un metabolito efector con una enzima metabólica, la cual, a su vez, interacciona o regula la actividad de proteínas reguladoras del desarrollo vegetal (proteínas con dominio MADS como NMH7) para activar programas de desarrollo considerando las necesidades ambientales.

Otra línea de investigación es el transporte núcleo-citoplásmico de las proteínas. En este trabajo se estableció que durante etapas muy tempranas del desarrollo de la plántula (semillas, plánutas de 1-2 días tras la germinación) el factor de transcripción NMH7 y la aldolasa forman un complejo citoplásmico. Por lo tanto, en este punto del desarrollo se podría analizar si la retención citoplásmica de ambas proteínas se debe a estados de fosforilación diferencial o bien, a la interacción con proteínas accesorias que los reclutan en el citoplasma. El mecanismo por el cual la aldolasa se trasloca al núcleo se desconoce incluso en los sistemas animales. Hasta el momento no se ha detectado una secuencia de localización nuclear (SLN) evidente. Sin embargo, la presencia de una SLN no es necesaria para facilitar el transporte de una proteína hacia al núcleo; así, la glucocinasa hepática no contiene un SLN, pero es transportada al núcleo mediante la interacción con proteínas reguladoras (Payne et al. 2005). Nuestros resultados indican que la interacción de la aldolasa con un factor de transcripción podría mediar su translocación al núcleo. Por otra parte, la presencia de la aldolasa en el núcleo de las células donde no se expresa NMH7 nos permitiría estudiar las interacciones diferenciales de esta enzima y dilucidar funciones alternas en un mismo organismo.

Conclusiones

- La presencia de NMH7 en plántulas y semillas no inoculadas sugiere que este factor de transcripción puede estar involucrado en eventos no simbióticos o en programas del desarrollo relacionado con la colonización de la bacteria.
- La fructosa-1,6-bisfosfato aldolasa citosólica clase I interacciona con NMH7, lo que indica un repertorio funcional más amplio para esta enzima glicolítica como se ha propuesto para otras enzimas metabólicas.
- Se dilucida que la función alternativa de la aldolasa es ligar el estado metabólico de la célula con las redes centrales del desarrollo.
- La aldolasa, al igual que otras enzimas metabólicas, desempeña funciones alternativas en el núcleo de las células vegetales.
- El fósforo y el nitrógeno regulan de manera sinérgica la expresión de NMH7 en etapas tempranas del desarrollo de *Medicago sativa*.
- Entre las posibles funciones de NMH7 se perfila la de ser un componente de la maquinaria que sensa las condiciones ambientales para ejecutar programas del desarrollo que conllevan al establecimiento de la simbiosis.
- La localización subcelular de NMH7 es regulada durante el desarrollo.
- El nitrato promueve la traslocación de NMH7 hacia las células no infectadas del nódulo simbiótico, por lo tanto estas células participan en la respuesta del nódulo al nitrato presente en el medio.

Apéndice 1

Hibridación *in situ* y localización subcelular de NMH7 en nódulos maduros de *Medicago sativa*

Antecedentes

Los patrones de expresión de los genes MADS box de plantas se han examinado generalmente a nivel de mARN usando hibridación *in situ*. Sin embargo, los patrones de expresión del mARN y su proteína no siempre coinciden debido posiblemente a modificaciones post-traduccionales o por el transporte de los polipéptidos a través de plasmodesmos. El transporte de factores de transcripción con dominio MADS entre células *via* plasmodesmos es un factor crítico para la determinación del destino celular.

El propósito de esta parte del trabajo de investigación es identificar el mARN de MNH7 empleando hibridación *in situ* para después correlacionarlo con la localización de su proteína empleando un anticuerpo específico generado contra la caja K y la región C-terminal.

Preparación de la sonda

El plásmido con la construcción pGEMTeasy NMH7 MIKC –el cual contiene clonado la secuencia codificante completa de NMH7– se empleó como templado para amplificar la caja K y la región C-terminal de NMH7. Los dos primers empleados fueron NMH7KF 5'CATGGAAGATGTCAATAG3' y NMH7CR 5' GGATCCGGGTGAGATCCGAG3'. Las condiciones de PCR fueron: 5 minutos de desnaturalización incial a 95° C, 30 ciclos de amplificación [30 segundos a 95° C (desnaturalización), 30 segundos a 60° C (alineamiento) y 30 segundos a 72° C (polimerización)]. Los productos amplificados de PCR se corrieron en un gel de agarosa al 1%, generando una banda de aproximadamente 400pb que se extrajo del gel empleando el sistema QIAquick Gel Extraction Kit (Inivitrogene). Para preparar la sonda antisentido de ADN de cadena sencilla marcada con digoxigenina, el producto de PCR purificado sirvió como templado para PCR unidireccional con el primer antisentido NMH7CR 5' GGATCCGGGTGAGATCCGAG3' en presencia de DIG-UTP (DIG-dUTP Labelling Mixture, Roche) empleando los mismos parámetros de PCR. El ADN de cadena sencilla marcado se purificó por el sistema QUIAquik Gel Extraction Kit (Invitrogen). La sonda se cuantificó empleando el sistema DIG High Prime DNA Labelling and Detection Started Kit II siguiendo las instrucciones del fabricante.

Hibridación *in situ*

Nódulos de 4 semanas se procesaron siguiendo el protocolo previamente descrito (Páez-Valencia et al. 2008 a). Los cortes transversales se desparafinaron y rehidrataron en alcoholes de concentraciones descendientes. Se incubaron durante 5 minutos con proteinasa K y se detuvo la reacción con una solución de PBS-Triton-Glicina (PBS, glicina 0.2 % Triton 0.1 %), se hicieron tres lavados con PBS-Tween. Las muestras se incubaron con solución de prehibridación (50mM PIPES pH 7.2, 0.75M de NaCl, 5mM EDTA, 100μg/mL de ADN de esperma de salmón, 0.1% de Ficoll 0.1 % polivinil pyrrolidine 40, 0.1% de BSA, 40 % de formamida desionizada) durante 1 hora a 70° C. Los cortes se trataron con Power Universal Reagent (HK085-5K) para evitar el pegado inespecífico. La muestras se incubaron con la sonda diluida en solución de hibridación durante toda la noche a 42° C. Los cortes se lavaron con un solución al 50 % de formamida en SSC 2×, luego se incouvaron con la misma solución durante 1 hora a 37° C. La reacción se paró adicionando una solución 1M de glicina. Las muestras se lavaron dos veces consecutivas con SSC 2× más dos lavados con PBS-Tween de 5 minutos cada uno a temperatura ambiente. Posteriormente, las muestras se incubaron con bloqueador de peroxidasas durante 5 minutos y se lavaron con PBS-Tween. Las muestras se sometieron a un bloque adicional por 1 hora y se incubaron con anticuerpo anti-digoxigenina acoplado a peroxidasa de rábano durante 1 hora a temperatura ambiente. Se hicieron tres lavados adicionales con PBS-Tween y se revelaron con un conjugado de diamino bencidina (DAKO). Los cortes se deshidrataron hasta xilol absoluto, se contrastaron con hematoxilina y se montaron con Entelan.

Inmunolocalización

Se utilizó inmunofluoresencia indirecta, empleando el anticuerpo específico contra NMH7 siguiendo el protocolo previamente descrito (Páez-Valencia et al. 2008b). Los cortes se visualizaron por microscopía confocal a temperatura ambiente.

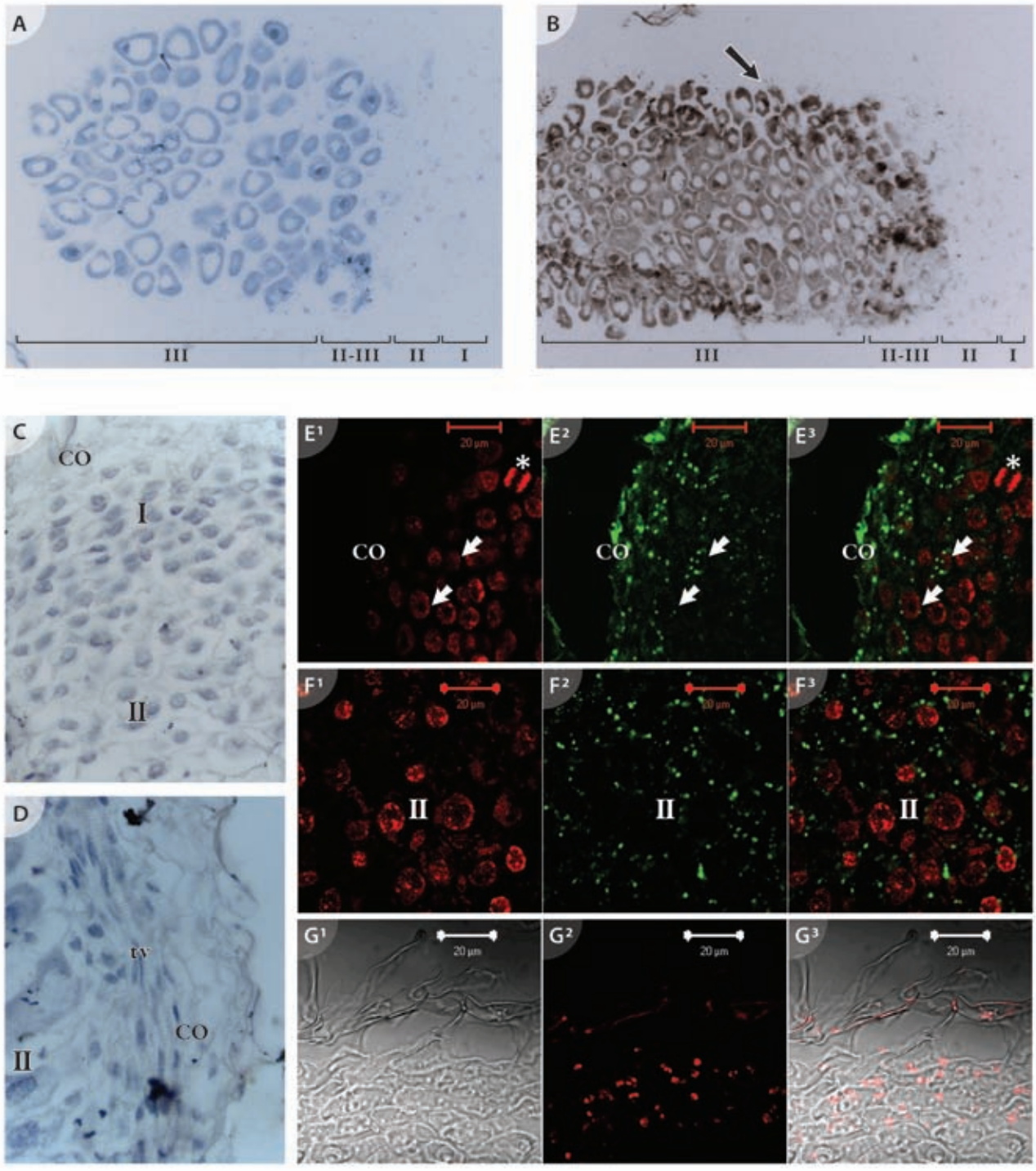

Figura 1. Hibridación *in situ* e inmunolocalización de NMH7 en nódulos maduros de *Medicago sativa.*

Hibridación *in situ:* A) Control negativo. B) Localización del transcrito en la interzona (II-III) y en la zona de fijación del N_2 (Zona III). C) No se detecta expresión en células del córtex contiguas a la región del apical del meristemo del nódulo ni en la Zona II. D) No se detecta expresión en tejido vascular.

Inmunolocalización. El canal rojo muestra los núcleos teñidos con ioduro de popidio; el canal verde, la localización de NMH7 empleando un anticuerpo secundario contra conejo acoplado a FITC (canal verde). E1-E3) Localización NMH7 en el citoplasma y núcleo de las células meristemáticas (flechas); el asterisco muestra la condensación cromosómica de células en anafase. F1-F3) Localización de NMH7 en las células de la Zona II presentando un patrón puntiforme. G1-G3) Localización nuclear de NMH7 en las células del córtex contiguas a las región meristemática empleando un anticuerpo secundario acoplado a rojo Texas (canal rojo).

Resultados

Aunque la expresión de NMH7 se ha descrito solamente a nivel de mARN, la aproximación empleada para predecir la localización de NMH7 en nódulos maduros de *Medicago sativa* no es concluyente: Se utilizó una sonda derivada de la caja MADS, la cual representa la región más conservada de estas proteínas y no es buena para discernir patrones específicos (Heard & Dunn 1995). En este trabajo empleamos la región codifcante de la caja K y región C-terminal como sonda antisentido y correlacionamos la expresión del NMH7 con la localización subcelular de su respectivo polipéptido. Para la descripción estructural de los nódulos, se empleó la nomenclatura propuesta por Vasse et al. 1990: zona I, meristemo; zona II, zona de infección; II-III interzona; zona III, zona fijadora de N_2. Los resultados del patrón del mARN son consistentes con los descritos previamente (Heard & Dunn 1995), es decir, no se detectó expresión en el meristemo, tejido vascular, córtex ni zona de infección (Zona II), (fig. 1 B, C, D) pero muestra una expresión generalizada en las células infectadas de la Interzona (II-III). (fig. 1 y 2 B) y de zona de fijación de N_2 (Zona III), excepto en la células no infectadas donde no se detectó el transcrito (fig. 2 B, H). El patrón de la proteína es consistente con el del

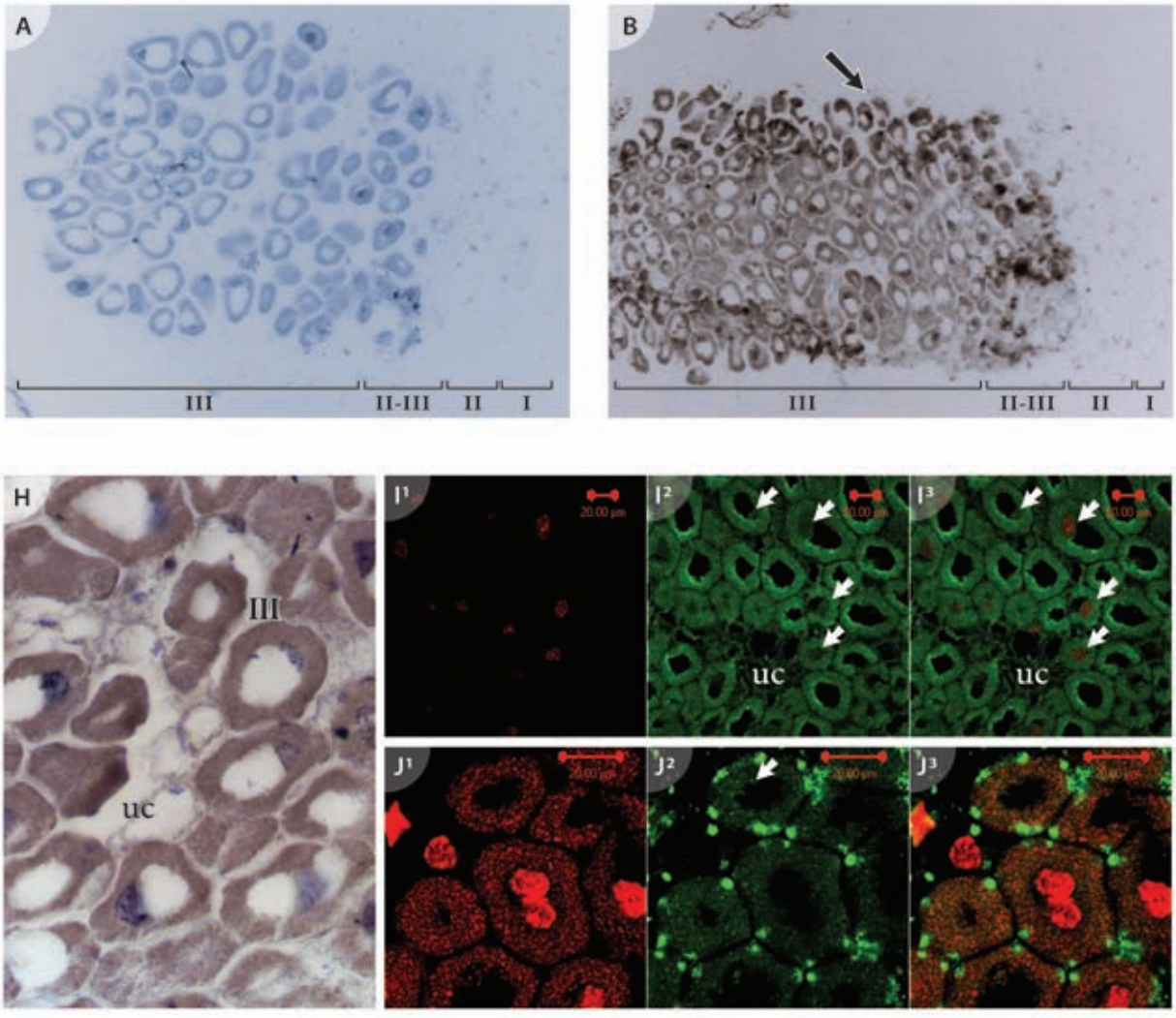

Figura 2. Hibridación *in situ* e inmunolocalización de NMH7 en nódulos maduros de *Medicago sativa.*

Hibridación *in situ:* A) Control negativo. B) Localización del *NMH7* en la interzona (II-III) y en la zona de fijación del N_2 (Zona III). H) En la zona III el NMH7 solamente se localiza en las células infectadas, no en aquéllas sin infectar.

Inmunolocalización: I1-I3) Localización citoplásmica y nuclear (flechas) de NMH7 en células infectadas; no se detecta la proteína en células no infectadas. J1-J3) Localización de NMH7 en los puntos de contacto entre las células ubicadas en la región adyacente al córtex.

mRNA, excepto por la localización de NMH7 en el núcleo de la capa interna del córtex (fig. 1 G1-G3), en el núcleo de las células merstiemáticas (fig. 1 E1-E3) y en el citoplasma de las células de la región II donde sí se detectó la presencia de la proteína presentado un patrón puntiforme (fig. 1 F1-F3). En la zona III, NMH7 se localiza en citoplasma y en el núcleo de las células infectadas (fig. 2 I1-I3) mas no se detectó su expresión en células no infectadas (fig. 2 I1-L3), sin embargo, presenta un patrón muy peculiar en los puntos de contacto entre células infectadas localizadas junto al parénquima (fig. 2 J1-J3). Un dato importante es que NMH7 es localizado exclusivamente en el núcleo de las capas de células que conforman el córtex del nódulo (fig. 1 G1-G3). Este fenómeno se ha descrito para algunos factores de transcripción; por ejemplo, la proteína SHR es localizada en el núcleo y en el citoplasma de las células del estele donde es producido y se desplaza hacia la endodermis, en la que se localiza exclusivamente en el núcleo. Esta observación es consistente con un modelo en el cual SHR queda atrapado en el núcleo de la endodermis a través de la interacción con otra proteína que causa su translocación a núcleo (Nakajima et al. 2001). Un dato interesante será corroborar la expresión de NGL9, una proteína MADS box con la que interacciona NMH7 *in vitro* y determinar su colocalización en las células corticales del nódulo similar a la interacción mostrada por sus ortólogos en *Arabidopsis* AP3/PI (McGonigle et al. 1996).

Apéndice 2

User : Mascot Daemon
Email :
Search title : Submitted from Ingel5857-5862 plante 20060606 by Mascot Daemon on RECHERCHE
MS data file : \\192.168.3.1\acquisitions\incoming\LTQ\plate281\Ingel5860.RAW
Database : UniRef100 (3511676 sequences; 1267845448 residues)
Taxonomy : Viridiplantae (Green Plants) (230657 sequences)
Timestamp : 6 Jun 2006 at 14:16:28 GMT
Significant hits: Q1SXG0 Fructose-bisphosphate aldolase, class-I [Medicago truncatula (Barrel medic)]
Q9SJQ9 Putative fructose bisphosphate aldolase [Arabidopsis thaliana (Mouse-ear cress)]
Q6RJ32 Fructose-bisphosphate aldolase [Pandanus amaryllifolius]
P43273 Transcription factor TGA2 [Arabidopsis thaliana (Mouse-ear cress)]

1. Q1SXG0 **Mass:** 38392 **Score:** 78 **Queries matched:** 7

Fructose-bisphosphate aldolase, class-I [Medicago truncatula (Barrel medic)]

Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
134	824.39	823.38	823.43	-0.04	0	23	1.9	1	K.VLAACYK.A
394	888.62	887.61	887.51	0.11	0	22	3.5	1	R.ALQQSTLK.A
695	972.52	971.51	971.54	-0.03	0	20	6.1	1	K.AAQEALLTR.A
1535	574.30	1146.59	1146.57	0.03	1	14	21	2	K.AWSGKEENVK.A
1572	577.76	1153.50	1153.56	-0.06	0	33	0.33	1	K.ANSEATLGTYK.G
1635	584.83	1167.65	1167.62	0.03	0	19	12	2	K.LGAGASESLHVK.D
2455	666.81	1331.61	1331.69	-0.08	0	76	1.4e-05	1	K.GILAADESTGTIGK.R

Mascot Search Results

User : Mascot Daemon
Email :
Search title : Submitted from Ingel5857-5862 plante 20060606 by Mascot Daemon on RECHERCHE

MS data file : \\192.168.3.1\acquisitions\incoming\LTQ\plate281\Ingel5861.RAW
Database : UniRef100 (3511676 sequences; 1267845448 residues)
Taxonomy : Viridiplantae (Green Plants) (230657 sequences)
Timestamp : 6 Jun 2006 at 14:28:29 GMT
Significant hits: Q1SXG0 Fructose-bisphosphate aldolase, class-I [Medicago truncatula (Barrel medic)]
Q9LF98 Fructose-bisphosphate aldolase [Arabidopsis thaliana (Mouse-ear cress)]
Q69V57 Putative fructose-bisphosphate aldolase [Oryza sativa (japonica cultivar-group)]
Q9AVH1 Putative senescence-associated protein [Pisum sativum (Garden pea)]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 37 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

1. Q1SXG0 **Mass:** 38392 **Score:** 127 **Queries matched:** 11

Fructose-bisphosphate aldolase, class-I [Medicago truncatula (Barrel medic)]

Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
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742	824.45	823.44	823.43	0.02	0	27	0.89	1	K.VLAACYK.A
814	829.51	828.50	828.38	0.13	0	(16)	16	1	K.YYEAGAR.F
815	829.56	828.55	828.38	0.18	0	19	7.4	1	K.YYEAGAR.F
1302	888.55	887.54	887.51	0.04	0	19	8	1	R.ALQQSTLK.A
1303	888.56	887.55	887.51	0.05	0	(17)	12	1	R.ALQQSTLK.A
1304	888.57	887.56	887.51	0.06	0	(10)	63	3	R.ALQQSTLK.A
1819	972.53	971.52	971.54	-0.02	0	21	4.7	1	K.AAQEALLTR.A
3389	744.93	1487.84	1487.79	0.05	1	81	4.5e-06	1	K.GILAADESTGTIGKR.L
3442	770.79	1539.56	1539.75	-0	1	59	0.000	1	K.ANSEATLGTYKGNSK.L

				0.19		3			
4085	740.34	2218.00	2218.07	-0.07	0	38	0.058	1	K.GTVELAGTDGETTTQGLDGLGAR.C

User : Mascot Daemon
Email :
Search title : Submitted from Ingel5857-5862 plante 20060606 by Mascot Daemon on RECHERCHE
MS data file : \\192.168.3.1\acquisitions\incoming\LTQ\plate281\Ingel5862.RAW
Database : UniRef100 (3511676 sequences; 1267845448 residues)
Taxonomy : Viridiplantae (Green Plants) (230657 sequences)
Timestamp : 6 Jun 2006 at 14:38:14 GMT
Significant hits: Q9FUG7 Cytosolic aldolase [Fragaria ananassa (Strawberry)]
Q1SXG0 Fructose-bisphosphate aldolase, class-I [Medicago truncatula (Barrel medic)]
P17815 Malate synthase, glyoxysomal [Ricinus communis (Castor bean)]

Q9FUG7 **Mass:** 38719 **Score:** 81 **Queries matched:** 1

Cytosolic aldolase [Fragaria ananassa (Strawberry)]

Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
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---

2. Q1SXG0 **Mass:** 38392 **Score:** 81 **Queries matched:** 2

Fructose-bisphosphate aldolase, class-I [Medicago truncatula (Barrel medic)]

Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
1017	486.87	971.73	971.54	0.19	0	81	2.2e-06	1	K.AAQEALLTR.A
2346	436.26	1305.77	1305.70	0.07	0	31	0.35	1	K.VAPEVVAEHTVR

User : Mascot Daemon
Email :

Search title : Submitted from 5863 plante 20060606 by Mascot Daemon on RECHERCHE
MS data file : \\192.168.3.1\acquisitions\incoming\LTQ\plate281\Ingel5863.RAW
Database : UniRef100 (3511676 sequences; 1267845448 residues)
Taxonomy : Viridiplantae (Green Plants) (230657 sequences)
Timestamp : 6 Jun 2006 at 14:23:12 GMT

Apéndice 3

Clonación de la fructosa-1,6-bisfosfato aldolasa citosólica clase I de *Medicago sativa*

La enzima Fructosa-1,6-bisfosfato aldolasa (ALD,EC4.1.2.13) es una enzima esencial en la glicólisis y la gluconeogénesis. La aldolasa cataliza la escisión aldólica de la fructosa-1,6-bisfosfato a dihidroxicetona fosfato y gliceraldehído 3-fosfato, así como la condensación aldólica reversa. En plantas, esta enzima también es un componente esencial de la ruta de las pentosas fosfato y el ciclo de Calvin (Flechner et al. 1999). Existen dos clases distintas de aldolasa: una independiente de iones metálicos (denominadas clase I) y otra que sí depende de ellos (clase II). Las plantas y animales solamente poseen aldolasas clase I, los procariontes y levaduras presentan aldolasas clase II, y ciertos organismos, como la *Euglena* (Protoctista fotosintético), presentan ambas. Las aldolasas clase I son homotetrámeros que forman una base de Shift con el sustrato y son inhibidas por borohidruros. Por otra parte, las clase II se encuentran como homodímeros, requieren de cationes divalentes como cofactores y son inhibidas por EDTA (Rutter et al. 1964). Las plantas poseen isoenzimas clase I localizadas en citoplasma y plastos. Ambas aldolasas, la citosólica (ALDc) y la plastídica (ALDp), son codificadas por genes nucleares distintos, los cuales se cree que evolucionaron a partir de la duplicación de un gene ancestral común (Plaxton 1996).

```
   1 M  T  N  F  K  S  K  Y  H  D  E  L  I  A  N  A  A  Y  I  G
   1 ATGACGAACTTCAAGAGCAAGTACCATGATGAGCTTATTGCCAATGCTGCCTACATCGGC
  21 T  P  G  K  G  I  L  A  A  D  E  S  T  G  T  I  G  K  R  L
  61 ACACCCGGCAAGGGTATTCTTGCTGCTGATGAGTCAACCGGAACAATTGGAAAGCGTCTA
  41 A  S  I  S  V  E  N  V  E  S  K  R  R  A  L  R  E  L  L  F
 121 GCTAGCATCAGCGTTGAGAATGTTGAATCCAAAAGACGTGCTCTCCGTGAACTCCTTTTC
  61 T  A  P  G  V  L  Q  Y  L  S  G  V  I  L  F  E  E  T  L  Y
 181 ACCGCCCCTGGTGTCCTTCAGTACCTTAGTGGAGTCATCCTCTTTGAGGAAACCCTCTAC
  81 Q  S  T  A  A  G  K  P  F  V  D  V  L  N  E  A  G  V  L  P
 241 CAAAGCACCGCTGCAGGCAAGCCTTTTGTTGATGTCTTGAACGAAGCTGGTGTGCTTCCT
 101 G  I  K  V  D  K  G  T  V  E  L  A  G  T  D  G  E  T  T  T
 301 GGTATCAAGGTTGACAAGGGTACCGTTGAGCTTGCCGGAACTGATGGAGAAACCACCACT
 121 Q  G  L  D  G  L  G  A  R  C  A  K  Y  Y  E  A  G  A  R  F
 361 CAGGGTCTTGATGGACTTGGTGCTCGTTGTGCTAAGTACTATGAAGCAGGTGCGCGTTTC
 141 A  K  W  R  A  V  L  K  I  G  P  N  E  P  S  E  H  S  I  H
 421 GCTAAATGGCGTGCAGTGCTTAAAATCGGCCCCAATGAGCCATCTGAGCACTCTATCCAT
 161 E  N  A  Y  G  L  A  R  Y  A  V  I  C  Q  E  N  G  L  V  P
 481 GAGAATGCCTATGGTTTGGCCCGATATGCAGTCATATGCCAAGAGAATGGACTTGTACCA
 181 I  V  E  P  E  I  L  V  D  G  P  H  D  I  Q  K  C  A  A  V
 541 ATTGTTGAGCCCGAGATCCTTGTTGATGGACCTCATGACATTCAAAAGTGTGCTGCTGTT
 201 T  E  R  V  L  A  A  C  Y  K  A  L  N  D  H  H  V  L  L  E
 601 ACCGAGCGTGTCCTTGCAGCATGCTACAAGGCCTTGAATGACCACCATGTCCTCCTTGAA
 221 G  T  L  L  K  P  N  M  V  T  P  G  S  D  A  P  K  V  A  P
 661 GGCACTCTCTTGAAGCCTAACATGGTTACCCCTGGATCTGATGCACCAAAGGTTGCACCC
 241 E  V  V  A  E  H  T  V  R  A  L  Q  R  T  V  P  A  A  V  P
 721 GAGGTTGTTGCTGAGCACACTGTTAGAGCTTTGCAGAGAACCGTACCTGCTGCAGTCCCA
 261 A  V  V  F  L  S  G  G  Q  S  E  E  E  A  S  V  N  L  N  A
 781 GCTGTTGTTTTCTTGTCTGGTGGACAGAGTGAGGAAGAGGCCAGTGTCAACCTCAATGCC
 281 I  N  Q  V  K  G  K  K  P  W  T  L  S  F  S  F  G  R  A  L
 841 ATCAACCAAGTCAAGGGTAAGAAGCCATGGACCCTTTCCTTCTCTTTTGGAAGGGCACTT
 301 Q  Q  S  T  L  K  A  W  S  G  X  E  E  N  V  K  A  A  Q  E
 901 CAACAGAGTACCCTCAAGGCATGGTCTGGAAANGAAGAAAATGTGAAGGCTGCTCAAGAA
 321 A  L  L  T  R  A  K  A  N  S  E  A  T  L  G  T  Y  K  G  N
 961 GCTTTGTTGACAAGGGCTAAGGCTAATTCTGAGGCTACTCTTGGAACTTACAAGGGTAAC
 341 S  K  L  G  A  G  A  S  E  S  L  H  V  K  D  Y
1021 TCTAAACTTGGTGCTGGTGCCTCAGAGAGTCTTCATGTTAAGGACTAC
```

Figura 1. Secuencia nucleotídica y peptídica deducida de la Fructosa 1,6-bisfosfato aldolasa citosólica clase I de *Medicago sativa*. Indicados por un recuadro, los codones de uso raro codificantes para Arginina (R) en *Escherichia coli*. La secuencia sombreada muestra la secuencia isoforma-específica seleccionada para la creación de anticuerpos policlonales en ratón.

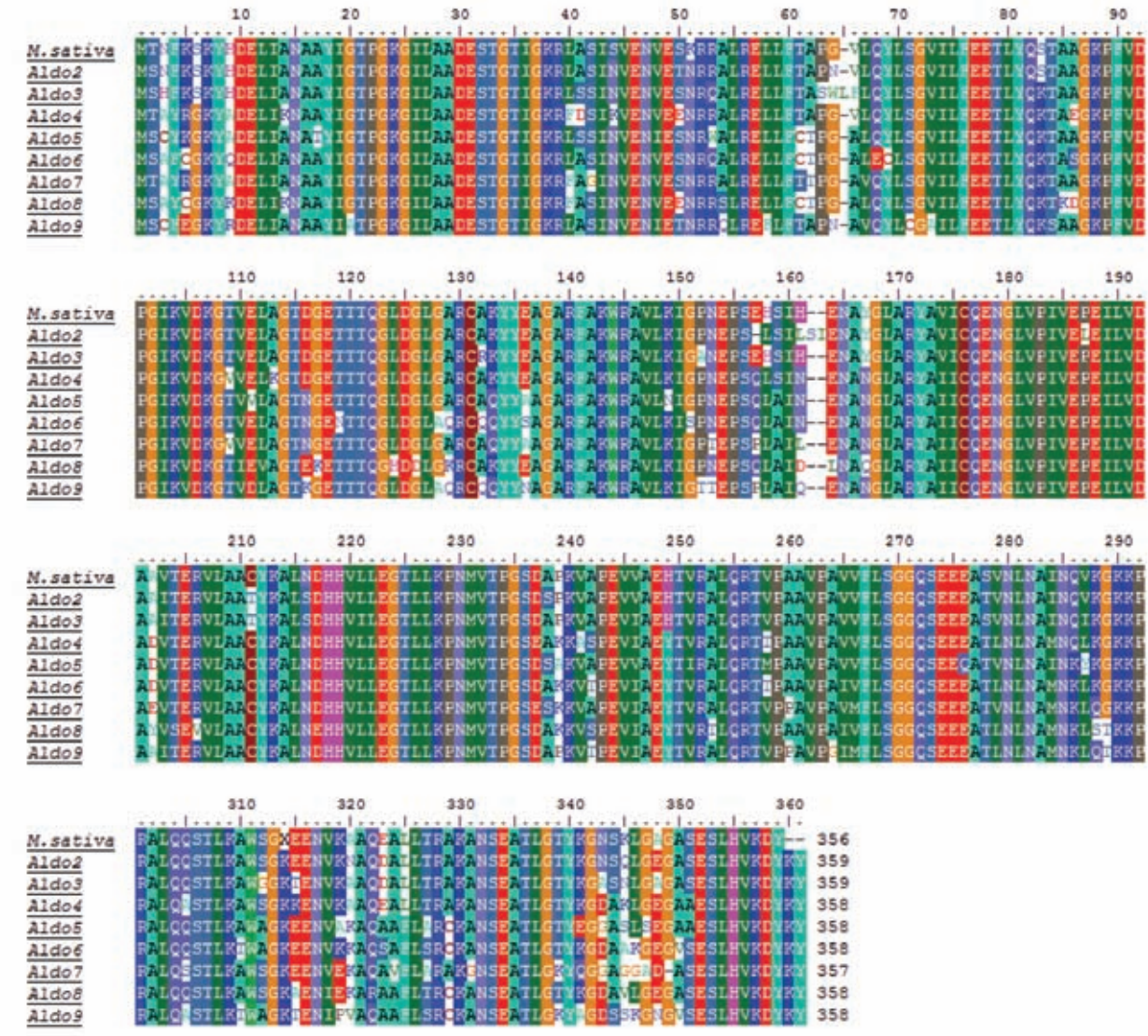


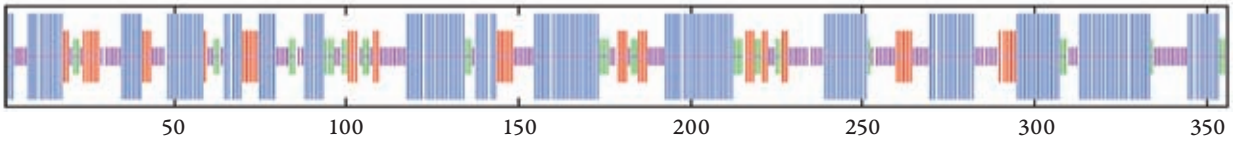
Figura 2. Alineamiento de la secuencia peptídica deducida de la Fructosa 1,6-bisfosfato aldolasa citosólica clase I de: *Medicago sativa;* Aldo 2: *Ciser arietinum;* Aldo 3: *Pisum sativum;* Aldo 4: *Fragaria ananassa;* Aldo 5: *Codonopsis lanceolada;* Aldo 6: *Persea americana;* Aldo 7: *Mesembtyanthemum crystallinum;* Aldo 8: *Oryza sativa;* Aldo 9: *Picea sitchensis.* Alinemamiento realizado con el programa Clustal W.

Estudios previos de inmunolocalización realizados en *Pisum sativum* demuestran que tanto la isoforma cloroplástica como la citosólica se localizan en el nucléolo y regiones de eucromatina sugiriendo un papel en la regulación de la expresión genética (Anderson 2005). Por otra parte, esta enzima interacciona con el factor de transcripción NMH7 en etapas tempranas del desarrollo de *Medicago sativa* (Páez-Valencia et al. 2008 b). Como parte de esta investigación se clonó la región codificante de la Fructosa 1,6-bisfosfato aldolasa citosólica clase I de *Medicago sativa*. Se diseñaron primers para amplificar la secuencia codificante de la aldolasa en base al alineamiento de las secuencias nucleotídicas y peptídicas de diversas aldolasas vegetales (datos no mostrados). Se amplificó la región codificante de la Aldolasa por PCR a partir de cADN de plántulas de *Medicago sativa,* entonces se clonó en un vector TOPO (Invitrogen) y el plásmido recombinante se amplificó en células DH5α. Se seleccionaron diversas clonas positivas y fueron secuenciadas. El marco abierto de lectura codifica un polipéptido de 356 aminoácidos (fig. 1) con un peso molecular deducido de 37.83 kDa. Un alineamiento múltiple demuestra que la secuencia deducida de aminoácidos comparte una similitud mayor al 90 % con las isoformas citosólicas de aldolasas reportadas en el GenBank para *Ciser arietinum, Pisum sativum, Fragaria ananassa, Codonopsis lanceolada, Persea americana, Mesembtyanthemum crystallinum,*

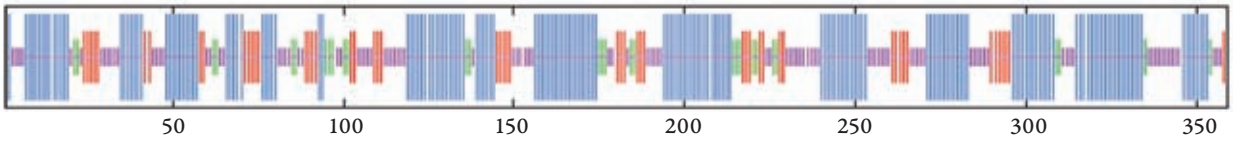
SOPMA:

Alpha helix	(Hh)
3_{10} helix	(Gg)
Pi helix	(Ii)
Beta bridge	(Bb)
Extended strand	(Ee)
Beta turn	(Tt)
Bend region	(Ss)
Random coil	(Cc)
Ambigous states	(?)
Other states	

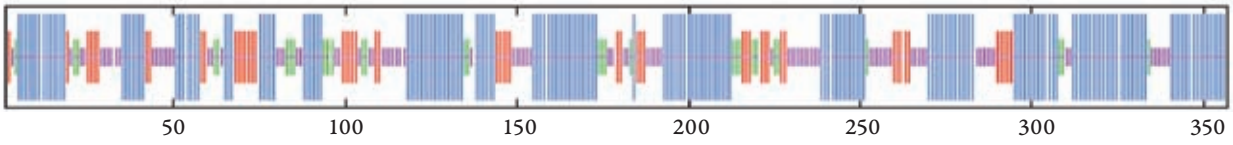
a) Medicago sativa



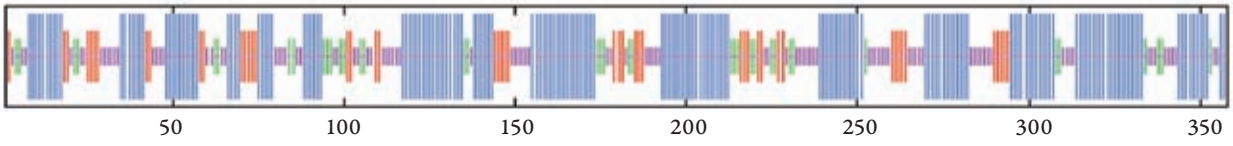
b) Pisum sativum



c) Mesembtyanthemum crystallinum



d) Oryza sativa



e) Picea sitchensis

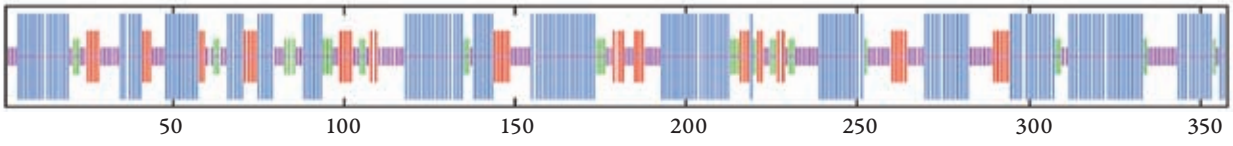


Figura 3. Comparación de la estructura secundaria de las Aldolasas citosólicas clase I de a) *Medicago sativa,* b) *Pisum sativum,* c) *Mesembtyanthemum crystallinum,* d) *Oryza sativa,* e) *Picea sitchensis.* El código de color para hélices, laminas, giros y rizos es indicado.

Oryza sativa y *Picea sitchensis* lo cual indica que clonamos la Fructosa-1,6-bisfosfato aldolasa citosólica clase I de *Medicago sativa* (fig. 2). El análisis de la estructura secundaria y el modelado molecular de la Furiosa-1,6-bisfosfato aldolasa citosólica clase I de *Medicago sativa* se realizó con el prgrama SOPMA. El análisis de la estructura secundaria revela que esta aldolasa está compuesta por 173 α-hélices, 34-giros β, articuladas por 55 cadenas extendidas y 108 rizos aleatorios (fig. 3).

Referencias

Flechner A., Gross W., Martin W., Schnarrenberger C.(1999) Chloroplast clas I and II aldolase are bifunctional for fructose-1,6-bisphosphate and sedoheptulose-1,7-bisphosphate cleavage in the Calvin cycle. FEBS Letters 447, 200-2002.

Rutter W. (1994) Evolution of aldolase. Federal Proceedings 22, 1248-1257.

Referencias bibliográficas

Armengaud P., Breitling R., Amtmann A. (2004) The potassium-dependendent transcriptome of Arabidopsis reveals a prominent role of jasmonic acid in nutrient signaling. *Plant Physiology* 136, 2556–2576.

Causier B., Cook H., Davies B. (2003) An *Anthirrinum* ternary complex factor specifically interacts with C-function and SEPALLATA-like MADS-box factors. *Plant Molecular Biology* 52, 1051–1061.

Chang D., Belaguli N., Roberts W., Iyer D., Wilmer B., Wu S., Dong X., Marx J., Moore M., Beckerle M., Majesky M,. Schwartz R. (2003) Cysteine-rich LIM-only proteins CRP1 and CRP2 are potent cardiovascular differentiation cofactors. *Developmental Cell* 4, 107–118.

Chi-Ham C. (2002) The DNA compacting protein DCP68 from soybean chloroplast is ferrodoxin:sulfite reducatase and co-localizes with organellar nucleoid. *Plant Molecular Biology* 49, 621–631.

Coen E., Meyerowitz E. (1991) The war of whorls: Genetic interactions controling flower development. *Nature* 353, 31–37.

Colebatch G., Ott G., Krusell L., Montanari O., Kloska S., Kopka J., Udvardi M. (2004) Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. *Plant Science* 39, 487–512.

Crawford K., Zambryski P. (2001) Non-targeted and targeted protein movement through plasmodesmata in leaves in different developmental and physiological states. *Plant Physiology* 125, 1802–1812.

David M., Deveran M., Batu J., Dedieu A., Domergue O., Chai J., Hertig C., Boistard P., Kahn D. (1998) Cascade regulation of nif gene expression in *Rizhobium meliloti*. *Cell* 54, 671–683.

de Folter S., Immink R., Kieffer M., Parenicova L., Henz S., Weigel D., Busscher M., Kooiker M., Colombo L., Kater M., Davies B., Angenet G. (2005) Comprehensive interaction map of the Arabidopsis MADS box transcription factors. *Plant Cell* 17, 1424–1433.

Desbrosses G., Kopka J., Udvardi M (2005) *Lotus japonicus* metabolic profiling: development of gas chromatography-mass spectrometry resources for study plant-microbe interactions. *Plant Physiology* 137, 1302–1318.

Franco-Zorrilla J., González E., Bustos R., Lindares F., Leyva A., Paz-Ares J. (2004) The transcriptional control of plant responses to phosphate limitation. *Journal of Experimental Botany* 55, 285–293.

Gonzalez J., York G., Walker G. (1996) *Rhizobium meliloti* exopolysacharides: synthesis and symbiotic function. *Gene* 179, 141–146.

Goto K., Meyerowitz E. (1994) Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. *Genes and Development* 8, 1548–1560.

Hassler M., Richmond T. (2001) The B-box dominats SAP-1-SRF interactions in the structure of ternary complex. *EMBO Journal* 15, 3018–3028.

Hirai M., Yano M., Goodenowe D., Kanaya S., Kimura T., Awazuhara M., Fujiwara T., Saito K. (2004) Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences USA* 101, 10205–10210.

Hong R., Hamaguchi L., Busch M., Weigel D. (2003) Regulatory elements of the floral homeotic gene AGAMOUS identified by phylogenetic footprinting and shadowing. *Plant Cell* 15, 1296–1309.

Honma T., Goto K. (2000) The Arabidopsis floral homeotic gene PISTILLATA is regulated by discrete cis-elements responsive to induction and maintenance signals. *Development* 127, 2021–2030.

Ichige A., Walker G. (1997) Genetic analysis of the *Rhizobium meliloti bacA* gene: functional interchangeability with the *Escherichia coli sbmA* gene en phenotypes of mutants. *Journal of Bacteriology* 179, 209–216.

Iyer D., Chang D., Marx J., Wei L., Olson E., Parmacek M., Balasubramanyam A., Schwartz R. (2006) Serum response factor MADS box serine-162 phosphorylation switches proliferation and myogenic gene program. *Proceedings of the National Academy of Sciences USA* 103, 4516–4521.

Kaufmann K., Melzer R., Theissen G. (2005) MIKC type MADS domain proteins structural modularity, protein interactions and network evolution in land plants. *Gene* 347, 317–326.

Lodwig E., Hosie A., Bourdes A., Findlay K., Allaway D., Karunakaran R., Downie J., Poole P. (2003) Amino acid cycling drives nitrogen fixation in the legumes *Rhizobium* symbiosis. *Nature* 422, 722–726.

Maathuis F., Filatov V., Herzyk P., Krijjer G., Axelsen K. (2003) Transcriptome analysis of root transporters reveals participation of multigene families in response to cation stress. *Plant Journal* 35, 675–692.

Maisero S., Imbriano C., Ravasio F., Favaro R., Pelucchi N., Gorla M., Mantovani R., Colombo L., Kater M. (2002) Ternary complex formation between MADS-box transcription factors and the histone fold protein NF-YB. *Journal of Biological Chemistry* 2777, 26429–26435.

McGonigle B., Bouhidel K., Irish V. (1996) Nuclear localization of the Arabidopsis APETALA3 and PISTILLATA homeotic gene products depends on their simultaneous expression. *Genes and Development* 10, 1812–1821.

Messenguy F., Dubois E. (2003) Role of MADS box protein and their cofactors in combinatorial control of gene expression and cell development. *Gene* 316, 1–21.

Miyake K., Ito T., Senda M., Ishikawa R., Harada T., Niizeki M., Akada S. (2003) Isolation of subfamily of gene for R2R3-MYB transcription factors showing up-regulated gene expression under nitrogen-limited conditions. *Plant Molecular Biology* 53, 237–245.

Nakajima K., Sena G., Nawy T., Benfey P. (2001) Intercellular movement of the putative transcription factor SHR in root patterning. *Nature* 413, 307–311.

Oke V., Long S. (1999) Bacteroid formation in *Rhizobium*-legume symbiosis. *Current Opinion in Microbiology* 2, 6641–6646.

Ott T., van Dongen J., Gunther C., Krusell L., Desbrosses G., Vigeolas H., Bock V., Czechowski T., Geigenberger P., Udvardi M. (2005) Symbiotic leghemoglobins are crucial for nitrogen fixation in legumes root nodules but not for general plant growth and development. *Current Biology* 15, 531–535.

Payne V., Arden C., Wu C., Lange A., Agius L. (2005) Dual role of phosphofructokinase-2/fructose bisphosphatase-2 in regulating the compartmentation and expression of glucokinase in hepatocytes. *Diabetes* 54, 949–1957.

Pelaz S., Tapia-López R., Álvarez-Buylla E., Yanofsky M (2001) Conversion of leaves into petals in Arabidopsis. *Current Biology* 11, 182–184.

Pellegrini L., Tan S., Richmond R. (1995) Structure of serum response factor core bound to DNA. *Nature* 376, 490–498.

Penmetsa R., Frugoli J., Smith L., Long S., Cook D. (2003) Dual genetic pathways controlling nodule number in *Medicago truncatula*. *Plant Physiology* 131, 998–1008.

Pramila T., Miles S., Thakurta D., Jemiolo D., Breeden L. (2002) Conserved homeodomain protein interact with MADS box protein Mcm1 to restricts ECB-dependent transcription to the M/G1 phase of the cell cycle. *Genes and Development* 16, 3034–3045.

Reichman J., Krizec B., Meyerowitz E. (1996) Dimerization specificity of Arabidopsis MADS domain homeotic protein APETALA1, PISTILLATA, APETALA 3 and AGAMOUS. *Proceedings of the National Academy of Sciences USA* 93, 4739–4798.

Sato N. (2001) The 70 kDa major DNA-compacting protein of chloroplast nucleoid is sulfite reducatse. *FEBS Letters* 487, 347–350.

Schulze W., Mann M. (2004) A novel proteomic screen for peptide-protein interactions. *Journal of Biological Chemistry* 279, 10756–10764.

Sepulveda J., Vlahopoulos S., Iyer D., Belaguli N., Schwartz R. (2002). Combinatorial expression of GATA4, Nkx2-5, and serum response factor directs early cardiac gene activity. *Journal of Biological Chemistry* 277, 25775–25782.

Spaink H. (2000) Root nodulation and infection factors produced by rhizobial bacteria. *Annual Review of Micriobiology* 54, 257–288.

Stouggard J. (2000) Regulation of legumes root nodules development. *Plant Physiology* 124, 531–540.

Theissen G., Seadler H. (2001) Floral quartets. *Nature* 409, 469–471.

Timmers A., Auriac M., Truchet G. (1999) Refined analysis of early symbiotic steps of the *Rhizobium-Medicago* interaction in relationship with microtubular cytoskeleton rearrangements. *Development* 226, 3617–3628.

Timmers A., Soupene E., Auriac M., de Billy F., Vasse J., Boistard P., Truchet G. (2000). Saprophytic intracellular *Rhizobia* in alfalfa nodules. *Molecular Plant–Microbe Interactions* 13, 1204–1213.

Trobner W., Ramirez L., Motte P., Hue I., Huijser P., Lonnig W., Seadler H., Sommer H., Schwarz-Sommer Z. (1992) *GLOBOSA:* a homeotic gene which interacts with DEFICIENS in the control of Antirrhinum floral organogenesis. *EMBO Journal* 11, 4693–4704.

Udvardi M., Kakar K., Wandrey M., Montanari O., Murray J., Andriankaja A., Zhang J., Benedito V., Hofer J., Chueng F., Town C. (2007) Legume transcription factors: global regulators for plant development and response to environment. *Plant Physiology* 144, 538–549.

Van de Wiel C., Scheres B., Franssen H., Van Lierop M., Van Lammeren A., Van Kammen A., Bisseling T. (1990). The early nodulin transcript ENOD2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules. *EMBO Journal* 9, 1–7.

Verdier J., Kakar K., Gallardo K., Le Signor C., Aubert G., Schlereth D., Udvardi M., Thompson R. (2008) Gene expression profiling of *Medicago truncatula* transcription factors identifies putative regulators of grain legumes filling. *Plant Molecular Biology* 67, 567–580.

Wang Z., Wang D-Z., Hockemeyer D., McAnally J., Nordheim A., Olson E. (2004). Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature* 428, 185–189.

Wasaki J., Yonetani R., Kuroda S., Shinano T., Yazaki J. (2003) Transcriptote analysis of metabolomics changes by phosphorus stress in rice plant roots. *Plant Cell and Environment* 26, 1515–1532.

Wintz H., Fox T. Wu Y., Feng V., Chen W., Chang H., Zhu T., Vulpe C. (2003) Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveals a novel transporters involved in metal homeostasis. *Journal of Biological Chemistry* 278, 47644–47635.

Wu X., Weigel D., Wigge P. (2002) Signaling in plants by intercellular RNA and protein movement. *Genes and Development* 16, 151–158.

Yahyaoui F., Küster H., Amor B., Hohnjec N., Puhler A., Becker A., Gouzy J., Vernié T., Gough C., Niebe A., Godiard L., Gamas P. (2004) Expression profiling in *Medicago truncatula* identifies more than 750 genes differentially expressed during nodulation, including many potential regulators of the symbiotic program. *Plant Physiology* 136, 3159–3176.

Zahn L., Leebens-Mack J., dePamhilis C., Theissen G. (2005) To B or not to B a flower: the role of DEFICIENS and GLOBOSA orthologs in the evolution of angiosperms. *Journal of Heredity* 96, 225–240.

Zhao M., New L., Kravchenko V., Kato Y., Gram H., di Padova F., Olson E., Ulvithc R., Han J. (1999) Regulation of the MEF2 family transcription factors by p38. *Molecular and Cell Biology* 19, 21–30.