UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

PROGRAMA DE DOCTORADO DE CIENCIAS BIOMEDICAS
CENTRO DE CIENCIAS GENÓMICAS

El nodo conformado por el microRNA 172c y su gen blanco, el factor de transcripción *APETALA2* (*AP2-1*), participa en la regulación de la simbiosis *Phaseolus vulgaris-Rhizobium etli*

TESIS
QUE PARA OPTAR POR EL GRADO DE:
DOCTORA EN CIENCIAS

PRESENTA:
BIOL. BARBARA NOVA FRANCO

TUTORA PRINCIPAL
DRA. GEORGINA HERNANDEZ DELGADO
CENTRO DE CIENCIAS GENÓMICAS UNAM

COMITÉ TUTORAL
DRA. MARIA DE LOURDES GIRAD CUESY
CENTRO DE CIENCIAS GENÓMICAS UNAM

DR. JOSE LUIS REYES TABOADA
INSTITUTO DE BIOTECNOLOGÍA UNAM

CUERNAVACA, MÉXICO, MAYO 2015

UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
CENTRO DE CIENCIAS GENÓMICAS

Tesis Doctoral:
"El nodo conformado por el microRNA 172c y su gen blanco, el factor de transcripción *APETALA2* (*AP2-1*), participa en la regulación de la simbiosis *Phaseolus vulgaris-Rhizobium etli*"
que para obtener el grado de Doctora en Ciencias presenta:
Bárbara Nova Franco

Este trabajo fue realizado en el laboratorio de la Dra. Georgina Hernández Delgado, del programa de Genómica Funcional de Eucariontes del Centro de Ciencias Genómicas/UNAM.

El comité tutoral que evaluó el avance del presente proyecto de investigación se compuso por los Dres. Georgina Hernández Delgado, María de Lourdes Girard Cuesy y José Luis Reyes Taboada. Además los Drs. Maheswara Pallavolu Reddy, y Mario Ramírez, fungieron como asesores externos a este comité.
El Jurado de la réplica oral de este trabajo de investigación se compuso por los Dres.Chirstian Sohlenkamp; Adriana Garay Arroyo; Esperanza Martínez Romero; María del Carmen Quinto Hernández, y Georgina Hernández Delgado.

BNF recibió una beca para estudios de doctorado y para estancia de investigación en el extranjero otorgada por CONACyT (351615). El trabajo experimental fue financiado por el Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT IN209710, IN202213, IN210814) de la Dirección General de Asuntos del Personal Académico (DGAPA) de la UNAM.

AGRADECIMIENTOS

Agradezco a todas y cada una de las personas que han estado conmigo cada de día de este largo proceso que es estudiar un Doctorado.

A mis padres, Lourdes Franco y Eugenio Nova por la paciencia, el amor y por todo lo que han hecho y sacrificado para que yo haya logrado estar en donde estoy.

A mis misses, Gina y Maluye, por todo el apoyo que me han brindado a lo largo de todos estos años, tanto en lo académico como en lo personal. Ya las estoy extrañando.

Al Dr. Jaime Mora Celis, por ser un hombre que me ha inspirado en muchos aspectos de mi vida. Por el tiempo que me he ha brindado para escucharme, hablarme y conocerme. Siempre será un ejemplo a seguir para mi.

A Pepe Reyes y Mahesh por todos los consejos para que mi trabajo de doctorado quedara mas bonito y mejor.

A mi grupo de investigación: Mario, Sarisa, Víctor, Damien, Enrique, Tania, Maru, gracias a todos y cada uno de ustedes por los buenos y malos momentos ya que me ayudaron a crecer como persona y como profesionista.

A Michael Udvardi, por la oportunidad brindada en su laboratorio, por ser un gran maestro. Por mover cielo, mar y tierra para poder regresar y ser parte de su grupo de trabajo.

A Luis Pedro, Luisito, muchas gracias por tu paciencia, por la buena disposición que siempre has tenido para ayudarme en todo lo que he necesitado y gracias por tu amistad incondicional.

A mi Alfonsi por todos los desayunos, las buenas platicas y la ayuda incondicional pero sobre todo por tu compañía.

A Jesús Arellano, gracias por ser el amigo que eres para mi. Por todos tus consejos y por siempre estar incondicional para mi.

A mis hermosas amigas y confidentes, Martha y Cinthya, gracias mil gracias por todas esas tardes de comida en su compañía, gracias por todas las risas y las lagrimas, por todas sus palabras de animo. Gracias por ser parte de mi vida.

A Adriana, porque siempre estuviste ahí para mi. Siempre me hiciste la vida ligera y me ayudaste en lo que pudiste. Muchas gracias.

A mi comadre hermosa, Marlene, gracias por ser quien eres para mi. Gracias por la amistad que me brindaste cuando mas la necesitaba. Gracias comadre por todos estos años y los que faltan de amistad.

A mi chulo y guapo amigo Donato, gracias por todas las discusiones científicas y no científicas, gracias por las peleas y por animarme a que publicara y a que hiciera y diera siempre lo mejor.

A mi mágica amiga Ofelia, gracias por ser quien eres y por haber llegado a mi vida en el momento correcto. Gracias por las platicas, por los ANOVAs, por las aventuras y sobre todo gracias por la magia.

A mis amigas y amigos de la Noble Foundation, ya que sin ustedes ese viaje hubiera sido impensable:

A Ana Páez García, porque te encontré sin haberte buscado. Por ser una gran amiga y maestra, por hacerme reír y secar mis lagrimas, por burlarte siempre que puedes de mi pero también por defenderme. Gracias por ser quien eres y por estar conmigo en esta nueva etapa de mi vida.

A Yanina Alarcón, por esos momentos maravillosos de amistad, por perdernos juntas siempre que salimos, por llorar y reír conmigo, por ser la única argentina que quiero que este siempre en mi vida.

A Ivone Torres, por tu ayuda y apoyo en el laboratorio y fuera de el. Gracias por estar a mi lado en un país diferente al de nosotras y gracias por encontrarnos.

A Raúl Huertas, porque a pesar de conocerte poco siento que te conozco demasiado. Gracias por hacer mi vida mas amena y mejor.

A Lia Kouri y a Catalina Pislariu por ser mis amigas y compartir su amistad conmigo.

Por ultimo, quiero agradecer a todas las personas que han estado conmigo siempre en mi vida y que se han alegrado por mis triunfos y entristecido por mis fracasos. A todos y cada uno de ustedes MUCHAS GRACIAS!!!

ÍNDICE:

CAPÍTULO I INTRODUCCIÓN

I.1 Importancia de las leguminosas y del frijol (*Phaseolus vulgaris*)

Las leguminosas son cultivos agronómicamente importantes a nivel mundial ya que son ricas en proteínas lípidos, fibras y diversos micronutrientes, por lo que son destinados al consumo humano y animal. Las leguminosas pertenecen a la familia Leguminosae también conocida como Fabaceae. Esta familia esta comprendida por 476 géneros y cerca de 14,000 especies, las cuales incluyen especies importantes para grano, pastura y producción de aceites. Los miembros de esta familia tienen una amplia distribución que abarca desde la zona ártica hasta las zonas tropicales (*Broughton et al., 2003*).

Las leguminosas aportan el 33% de proteína a la dieta de los humanos. Las leguminosas con mayor aporte de proteína son: frijol, chícharo (*Pisum sativum*), garbanzo (*Cicer arietinum*), “cowpea” (*Vigna unguiculata*) y lentejas (*Lens culinaris*). Además de su aporte nutricional, las leguminosas son importantes debido a su producción de aceites vegetales principalmente la soya y el cacahuate. Por otro lado, existen leguminosas forrajeras, las cuales son necesarias para el mantenimiento de la salud animal, como la alfalfa (*Medicago sativa*) (*Graham y Vance 2003*).

Además de ser cultivos importantes por su nivel nutricional, las leguminosas pueden formar asociaciones simbióticas con diferentes bacterias de los géneros *Rhizobium, Mesorhizobium, Sinorhizobium, Bradyrhizobium* y *Azorhizobium* (colectivamente llamadas como rhizobia). Estas bacterias interactúan con las plantas leguminosas para formar ciertos órganos especializados denominados nódulos, donde se lleva a cabo la fijación de nitrógeno atmosférico. Las leguminosas noduladas ofrecen una ventaja selectiva en suelos sin abonar ya que pueden crecer bien en estas zonas donde no lo harían otras plantas. Por otro lado, proveen a los suelos con nitrógeno para que otras plantas puedan utilizarlo, como es el caso de

la rotación de cultivos de maíz cuando se cultiva en suelos en donde previamente fue cultivada la alfalfa (*Graham y Vance 2003*).

Además de las asociaciones simbióticas que forman con el genero *Rhizobium*, las leguminosas pueden formar asociaciones endosimbioticas con los hongos arbusculares formando lo que se conoce como micorrizas arbusculares (AM). Las micorrizas pueden asociarse con la mayoría de las plantas existentes y tienen como principal función la adquisición de nutrientes como el fósforo. En esta asociación simbiótica la planta le provee al hongo compuestos carbonados para su crecimiento y desarrollo mientras que el hongo transfiere agua y nutrientes, como fósforo, a la planta a través de sus hifas que conectan el suelo con las células vegetales, lo que tiene como ventaja que las raíces micorrizadas, por medio de las hifas de los hongos, tengan una mayor área de absorción y exploración en el suelo (*Miransari 2014*).

En México, una de las leguminosas de gran importancia es el frijol (*Phaseolus vulgaris*); la producción de esta planta se destina en forma directa al consumo humano, representando una de las principales fuentes de proteína para amplios sectores de la población mexicana. En una buena parte de América Latina, el cultivo de frijol, junto con el del maíz representa toda una tradición productiva y de consumo, cumpliendo diversas funciones de carácter alimentario y socioeconómico que le han permitido trascender hasta la actualidad. Su presencia a lo largo de la historia lo ha convertido no solo en un alimento tradicional, sino también en un aspecto de identificación cultural. Se trata de uno de los cultivos de mayor importancia, ya que representa para la economía de los productores una fuente importante de ocupación e ingreso, a la vez que es una garantía de seguridad alimentaria. En el ámbito mundial, México se encuentra entre los cinco principales productores de frijol (7% en promedio de las cosechas mundiales) conjuntamente con la India, Brasil, China y los Estados Unidos de América, países que en total aportan el 63% de la producción mundial (*Paredes et al., 2006*).

Con respecto a la demanda de grano de frijol en México, durante la década de los noventa se estimó que el 89% se canalizó al consumo humano, el 9% se utilizó como semilla y

el resto fueron perdidas. A finales del siglo XX, en México se consumieron cerca de 2.2 millones de toneladas, destinándose el 41% de la producción al abastecimiento de la población rural; el restante 59% se empleó para satisfacer el consumo de la población urbana (*Paredes et al., 2006*).

I.2 Simbiosis Rhizobium-Leguminosa

I.2.1 Primeras etapas de la nodulación

Las interacción del rhizobia con las raíces de las leguminosas comienza con la secreción de flavonoides por parte de las raíces y la consecuente producción de los factores de nodulación (Factores Nod (NF), lipoquitooligosacáridos) por parte de las rhizobia. Los NF producidos por las rhizobia poseen un esqueleto de quitooligosacáridos que esta compuesto generalmente por cuatro o cinco residuos N-acetilglucosamina (GlcNAc) unidos por enlaces β1-4. Estos lipoquitooligosacáridos pueden ser posteriormente modificados por una gran variedad de sustituyentes en las subunidades de GlcNAc como pueden ser grupos de metil, fucosil, acetil y sulfato. Estas modificaciones pueden variar entre los NF producidos por diferentes especies de rhizobia; y son importantes para la especificidad de la leguminosa hospedera con su rhizobia compatible (*Oldroyd 2013*). Los genes de rhizobia responsables de producir a los NF son conocidos como genes *nod;* existen diferentes tipos entre ellos se encuentran los denominados: *nodA, nodB, nodC* y *nodD*. El operon *nodABC* es el responsable de la biosíntesis del esqueleto de quitina de los NF mientras que *nodD* es el regulador transcripcional que activa a los anteriores genes *nod.* Existen además otros genes *nod* los cuales son responsables de las modificaciones generales de la estructura de los NF. (*Shamseldin 2013*)

Existen varios reportes en los cuales se han identificado los genes de las leguminosas involucrados en la activación de la respuesta mediada por NF. Los NF producidos por los rhizobia son percibidos por las células epidermales de los pelos radicales por receptores de tipo cinasas con dominios LysM en su región extracelular conocidos como: Nod Factor Receptor 1/LYSM

Domain Containing Receptor-Like Kinase 3 (*NFR1*/*LYK3*, *NFR5*/*NFP*). En diferentes leguminosas como *Medicago truncatula, Lotus japonicus* y soya (*Glycine max*), estos factores han sido caracterizados (Murray 2011) y se han caracterizado en dos grupos: los pertenecientes al clado de LysM I, el cual incluye al Nod Factor Receptor 1 (NFR1) y LysM Receptor Kinase 3 (LYK3) de *L. japonicus* y *M. truncatula* respectivamente; y los pertenecientes al clado de LysM II, en donde se encuentran NFR5 y Nod Factor Perception (NFP) de *L. japonicus* y *M. truncatula* respectivamente (Oldroyd 2013).

El reconocimiento de los factores Nod desencadena varias respuestas en las células epidermales de la raíz de las leguminosas como son: una cascada de fosforilación y la generación de flujos de calcio en el citoplasma de la célula (*Murray 2011*), esto flujos de calcio generan la despolarización de la membrana nuclear que activa canales de calcio modulados por voltaje y permite la entrada de calcio al núcleo, el calcio se une y activa a la proteína cinasa dependiente de calcio-calmodulina (*CCaMK*). La *CCaMK* activada interacciona con la proteína *IPD3* (*CYCLOPS*) y el dímero CCaMK/CYCLOPS a su vez interacciona con los factores de transcripción *NSP1* y *NSP2* (NODULATION SIGNALING PATHWAY) que induce la expresión de otros factores de transcripción como: *NY-FA1/NY-FA2* (Nuclear Factor A1/A2) *ERN1* (Ethylene Responsive Factor REQUIRED FOR NODULATION) y *NIN* (NODULE INCEPTION) (*Oldroyd 2013*)(Fig. 1)

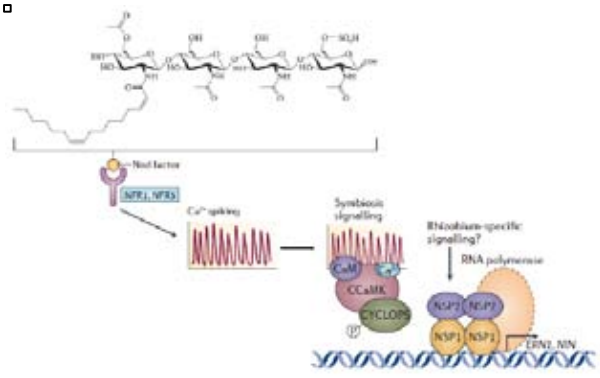


Figura 1. Reconocimiento de los factores Nod por los receptores NFR1/5 y la señalización rio abajo de las oscilaciones de calcio en el proceso simbiótico. Después del reconocimiento de los NF por parte de los receptores NOD FACTOR RECEPTOR 1/5 (NFR1/5), la percepción y transducción de la señales de calcio involucra a la proteina Ca^{+2}/ CALMODULIN-DEPENDENT PROTEIN KINASE (CCaMK), que interactua con CYCLOPS, y otros componentes rio abajo, como los reguladores trascripcionales NODULATION SIGNALING PATHWAY (NSP1/NSP2), controlan la expresión de los genes tempranos de nodulación como ETHYLENE-RESPONSIVE FACTOR REQUIRED FOR NODULATION (ERN1) y NODULE INCEPTION (NIN). (Modificado de *Oldroyd 2013*).

Cuando el NF es percibido por los NFRs se inicia un encorvamiento de los pelos radicales de la raíz, “atrapando” a la bacteria en su interior. Posteriormente se comienzan a expresar diferentes genes relacionados con la formación del nódulo, conocidos comúnmente como nodulinas tempranas (ENODs) las cuales producen un rearreglo del citoesqueleto de las células epidermales formando una especie de túnel llamado hilo de infección, el cual se forma por medio de la invaginación de la pared celular y la membrana plasmática cerca de la punta de las células epidermales del pelo radical (Fig. 2) (Udvardi y Poole 2013). A través del hilo de infección los rhizobia avanzan hacia la zona de las células corticales en donde una vez formado el nódulo se diferenciara en un bacteroide capaz de fijar nitrógeno atmosférico. Este hilo de infección va desde la punta del pelo radical hasta la zona de las células corticales, en la cual concomitantemente se ha iniciado un proceso de división celular, dando origen al primordio celular que finalmente formara al nódulo (*Oldroyd y Downie 2008*).

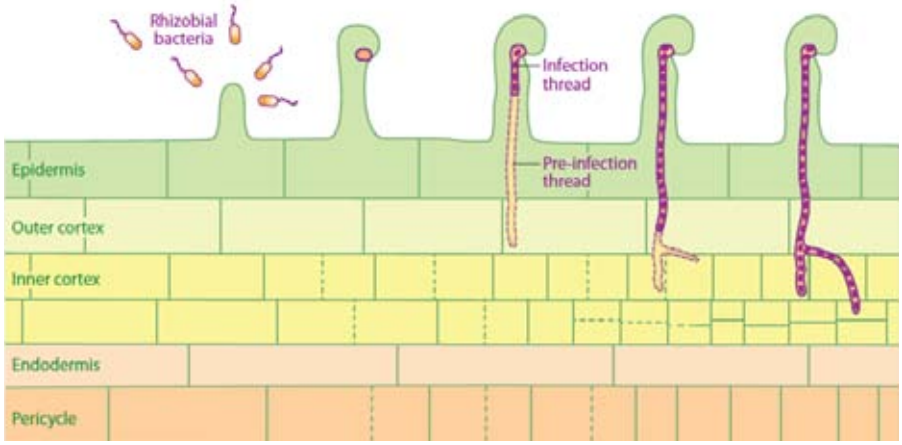


Figura 2. Proceso de infección en la simbiosis *Rhizobium*-Leguminosa. Las bacterias son atrapadas en el pelo radical encorvado y a partir de ahí, se forma el hilo de infección, este progresa hasta las células del córtex interno donde el primordio del nódulo es formado a través de una serie de divisiones celulares. (Tomado de *Oldroyd et al., 2011*).

En el desarrollo del hilo de infección participan otros elementos, en adición a los antes mencionados. Actualmente se ha descrito la función de dos flotilinas (*FLOT2* y *FLOT4*), las cuales se requieren para la iniciación del hilo de infección y que poseen características de balsas lipídicas, en *M. truncatula*; estas son consideradas compartimentos especializados de la

membrana plasmática de las células de la raíz y han sido asociadas con funciones de tipo receptor necesarias para el reconocimiento y señalización de los NF (Oldroyd et al., 2011).

Se ha observado que el silenciamiento de ellas provoca una disminución del hilo de infección, lo cual sugiere su papel en el proceso de infección; además, se sabe que su expresión es dependiente de *NIN* y de *NSP2* (*Murray 2011*). Durante el proceso de nodulación, varios genes de la planta son inducidos, lo cual sugiere que son importantes para la simbiosis rhizobia-leguminosa. Uno de estos genes es *ENOD40*, el cual ha sido reportado como una nodulina temprana. Aún no se conoce si este gen codifica para un péptido o para un RNA que no codifica para proteína (ncRNA). Lo que se sabe es que se expresa principalmente en las células del periciclo y que tiene la posible función de activar el ciclo celular para la formación del primordio del nódulo ya que después se ha visto su expresión en estas células (*Fergurson 2010*).

I.2.2 Autorregulación de la nodulación

La formación y el mantenimiento de los nódulos provoca un gasto energético por parte de la planta, la cual ha desarrollado mecanismos para optimizar la cantidad del número de nódulos y la optima fijación de nitrógeno, por medio de un proceso denominado: Autorregulación de la Nodulación (AON). La AON es un proceso sistémico y se desencadenada después de los primeros eventos de nodulación, se ha predicho que comienza con la producción de péptidos *CLE* (CLAVATA-ESR-relates) en la raíz de las leguminosas. En soya, han sido identificados dos péptidos *CLE* llamados *Rhizobium*-Induce *CLE1* y *CLE2* (*GmRIC1* y *GmRIC2*) (*Reid et al., 2011*) que poseen un patrón de expresión diferente; mientras que *RIC1* se expresa en la raíz a tiempos muy tempranos después de la infección (12 horas); *RIC2* se expresa después de 72 horas (*Reid et al., 2013*). En soya, estos péptidos *CLE*, se transportan hasta la parte aérea en donde actúan como ligandos del receptor de cinasa LRR (Leucine-Rich Repeat) CLAVATA1-like denominado Nodulation Autoregulation Receptor Kinase (*GmNARK*) (*Okamoto et al., 2009*). Se ha propuesto que la activación de este receptor en la parte área da inicio a la producción del

inhibidor SDI (shoot-derived inhibitor). SDI es una molécula pequeña cuya naturaleza aún no ha sido identificada pero que es transportada hasta las raíces en donde se encarga de inhibir la formación de más nódulos (Fig. 3) (*Magori y Kawaguchi 2009*). Se ha reportado un mecanismo de AON smilar en otras leguminosas como *L. japonicus* y *P. vulgaris* (Ferguson et al., 2014).

Además de las señales internas de la planta para controlar el número de nódulos, las señales externas, como la cantidad de nitrógeno en el suelo, se ha considerado como importante en el control de la formación de nódulos. Se ha reportado desde hace años que niveles altos de nitrógeno inhiben la formación de nódulos en la raíces (*Streeter y Wong 1988*). Trabajos recientes indican que la inhibición de la nodulación por nitrato puede llevarse a cabo por medio de un tipo de péptidos *CLE*, llamados Nitrate Induced CLE peptides (*NIC*), que son percibidos por el receptor de tipo LRR RK (*GmNARK*) en la raíz, lo cual tiene como consecuencia la inhibición de la formación de los nódulos, este proceso no se considera sistémico debido a que se lleva a cabo solo en la raíz (*Okamoto et al., 2009; Reid et al., 2011; Soyano et al., 2014*) (Figura 3).

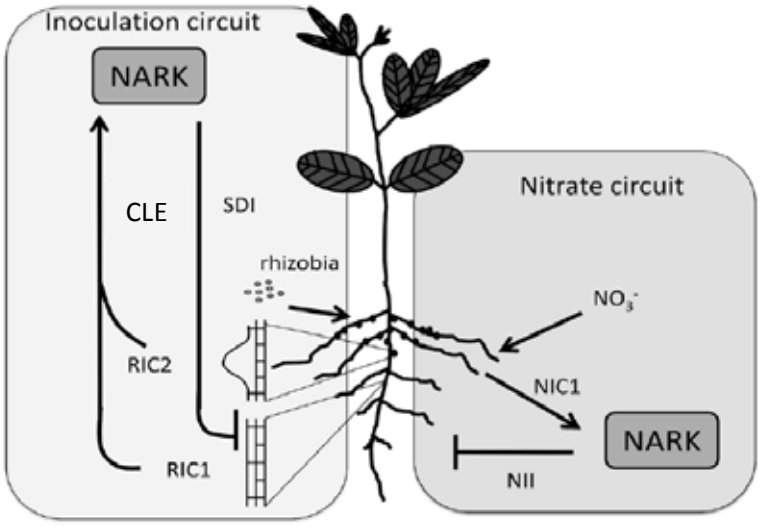


Figura 3. Proceso de Auto Regulación de la Nodulación (AON) inducida por *Rhizobium* y por nitrato. Las leguminosas regulan la nodulación en respuesta a eventos de infección pre-existente por medio de los péptidos CLE Rhizobium-Induced (RIC1 y RIC2) y por medio de los péptidos Nitrate-Induce (NIC1) en respuesta a nitrato. La AON en respuesta a *Rhizobium* es vía sistémica a través de el receptor NODULE AUTOREGULATION RECEPTOR KINASE (NARK) en la parte área mientras que la regulación mediada por nitrato se lleva a cabo de manera local en las raíces de las leguminosas. (SDI, Shoot-derived inhibitor; NII, Nitrate Indiced Inhibitor) (Tomado de *Reid et al., 2011*)

I.2.3 Fijación simbiótica de nitrógeno y asimilación de amonio

Por medio del hilo de infección las rhizobia son liberadas dentro de las células vegetales a los simbiosomas, los cuales son porciones de la membrana celular vegetal que rodea individualmente o en pequeños grupos a las bacterias las cuales se convierten en unas formaciones ramificadas, hinchadas y deformes llamadas bacteoides. Los bacteroides pueden ser de dos tipos: en los nódulos indeterminados, como los de *M. truncatula*, los bacteroides de *Sinorhizobium meliloti* experimentan una endoreduplicación lo que tiene como consecuencia una cuenta de 24 cromosomas comparado con uno a dos cromosomas que poseen en vida libre; mientras que en los nódulos determinados, como los de *L. japonicus* y *P. vulgaris*, los bacteroides mantienen uno a dos cromosomas y pueden ser crecidos del mismo nódulo de nuevo, lo que no ocurre con los bacteroides de los nódulos indeterminados (Oldroyd et al., 2011). La fijación de nitrógeno no se inicia hasta que se han formado los simbiosomas y se requiere, además, de la interacción coordinada de dos clases de genes presentes en las rhizobia: los genes *nif* y los genes *fix.* Los genes *nif* codifican para la enzima nitrogenasa la cual posee dos subunidades proteicas, una de Fierro-Molibdeno (FeMo) y otra solo de Fierro (Fe). Estas dos subunidades son codificadas por los genes *nifK* y *nifD* (subunidad FeMo); y *nifH* (subunidad Fe). La regulación de estos genes *nif* es controlada por *nifA* (un activador transcripcional positivo) y *nifL* (regulador negativo). Todos los genes *nif* son regulados por condiciones ambientales tales como oxígeno y nitrógeno. Por ejemplo, altas concentraciones de amonio en el suelo permite que *nifL* actué como un regulador negativo, reprimiendo la expresión de *nifA* (*Shamseldin 2013*).

Además de los genes *nif* existen otro grupo de genes involucrados en la fijación de nitrógeno que colectivamente son llamados genes *fix* los cuales responden a bajas concentraciones de oxígeno (Udvardi y Poole 2013). El genero *Rhizobium* pertenece a la clasificación de organismos aeróbicos estrictos y necesita de O_2 para generar la energía a través de la respiración, la cual utiliza para la fijación de nitrógeno; sin embargo, su nitrogenasa es

inactivada por el O_2; lo anterior se conoce como la “paradoja del oxígeno” (*Girard et al., 2000*). La nitrogenasa que se encuentra en los nódulos posee dos características principales: sensibilidad al O_2 y la capacidad de reducir N_2 y acetileno. En el nódulo, las concentraciones de O_2 son muy bajas pero, a pesar de ello, la respiración por *cbb3* (citocromo oxidasa terminal) codificada por el operon *fixNOQP* permite al bacteroide producir suficiente energía para poder fijar nitrógeno. Esta oxidasa posee como principal característica su extrema afinidad por O_2 (*Marchal y Vanderleyden 2000*) ya que en el nódulo existe un ambiente microaeróbico. Existen tres procesos que intersectan para que en el nódulo se lleven a cabo las condiciones microaeróbicas necesarias para el buen funcionamiento de la enzima nitrogenasa: una barrera de difusión gaseosa en las capas celulares externas del nódulo que limita el paso de oxígeno al interior de las células infectadas; las mitocondrias tanto de los bacteroides como de las plantas, las cuales poseen tasas de respiración muy altas y consumen el oxígeno tan rápido como entra a los nódulos y por ultimo las hemoglobinas de plantas, conocidas como leghemoglobinas que poseen una alta afinidad al oxígeno (Udvardi y Poole 2013). La leghemoglobina es una proteína que contiene hierro y que esta siempre presente en los nódulos sanos fijadores de N_2 dándoles un color rojo y funciona como “regulador de O_2”, cuyo ciclo va de la forma oxidada del hierro (Fe^{3+}) a la reducida (Fe^{2+}) para mantener la concentración optima de oxígeno en el interior del nódulo que es baja pero constante (*Shamseldin 2013*).

El primer producto estable que se obtiene en la fijación de nitrógeno es el amonio que se produce en los nódulos por las bacterias; sin embargo, varios tipos de experimentos indican que, la asimilación del mismo en los nódulos para formar compuestos de nitrógeno orgánico, la lleva a cabo principalmente la planta ya que tiene elevadas concentraciones de glutamino sintetasa la cual cataliza la reacción de glutamato y amonio para formar glutamina. Aúnque también se ha visto que los bacteroides pueden asimilar algo de amonio y formar compuestos orgánicos ya que poseen unos niveles muy bajos de enzimas asimiladoras de amonio. Por tanto, el amonio transportado desde el bacteroide a la célula vegetal es asimilado por la planta

sintetizando el aminoácido glutamina. A partir de la glutamina en las células vegetales del nódulo se sintetizan diferentes compuestos nitrogenados que luego son transportados a los tejidos vegetales. En las leguminosas que forman nódulos indeterminados como *M. truncatula* los compuestos transportados son amidas, como la asparagina y la 4-metilglutamina. Por otro lado, en leguminosas que forman nódulos determinados como lo es frijol y soya, los compuestos nitrogenados transportados son ureidos como alantoina y ácido alantóico (*Smith y Atkins 2007*).

En los nódulos determinados de frijol, el amonio es asimilado en glutamina en las células infectadas y el amonio se utiliza para la síntesis de purinas. Las purinas después son oxidadas dentro del citosol de las células infectadas a uratos los cuales después son transferidos a las células no infectadas para posteriormente ser oxidados en alantoina y ácido alantóico (ureidos). Los ureidos son exportados del nódulo al xilema para que la planta pueda utilizarlos en su nutrición como fuente de N (Figura 4).

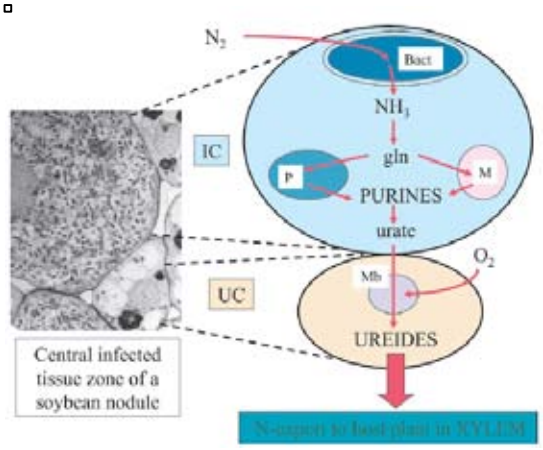


Figura 4. Asimilación de nitrógeno en nódulos determinados. La asimilación de amonio en nódulos determinados tiene como principal producto la glutamina, en las células infectadas (IC), la cual sirve como sustrato para la síntesis de purinas, las cuales posteriormente son oxidadas en forma de uratos para producir ureidos dentro de las células no infectadas (UC) (Tomado de *Smith y Atkins 2007*).

I.2.4 Senescencia de nódulos

Después de la fijación de nitrógeno y con el paso del tiempo los nódulos determinados comienzan a envejecer en un proceso conocido como senescencia de nódulos. Este proceso

es visible por el cambio de color de rosa (asociado con la funcionalidad de la proteína leghemoglobina) a verde (asociado con la degradación del grupo hemo) (*Roponen 1970*). A nivel estructural, se producen diversos cambios como es que el citoplasma ya no es tan denso y se comienza a desintegrar la membrana del simbiosoma (*Timmers et al., 2000*). Una característica distintiva del proceso de senescencia, es la actividad proteolítica que provoca la degradación a gran escala de proteínas, lo que produce como resultado final la muerte tanto de los bacteroides como de las células del nódulo (*Van de Velde et al., 2006*).

La senescencia de los nódulos está positivamente regulada por etileno. *Van de Velde et al.,* (2006) reportaron haciendo un perfil transcriptómico de *M. truncatula*, una inducción de genes involucrados en la síntesis de etileno como el gene ACCO (1-aminociclopropano-1-carboxilato oxidasa) en la zona de senescencia de nódulos.

I.3 Los microRNAs como reguladores post-transcripcionales en plantas

Los RNAs pequeños de eucariotes (sRNA) de 20-30nt de longitud, constituyen una familia de RNAs que no codifican para proteínas y que participan en procesos regulatorios a través de diferentes mecanismos tales como: corte endonucleotílico del RNA mensajero blanco, represión traduccional y represión transcripcional a través de modificación del DNA y/o cromatina (*Chen 2009*).

Existen diferentes clasificaciones de los RNAs pequeños en plantas, la mayoría basados en su biogénesis. Una de las clasificaciones más conservadas divide a los RNAs pequeños en dos grandes grupos: los microRNAs (miRNAs) y los RNAs pequeños de interferencia (siRNAs). Estos pueden distinguirse por su modo de acción y su biogénesis ya que, aúnque ambos pueden inducir el corte del mRNA blanco y la represión traduccional, pero solo los siRNAs pueden actuar a nivel de modificaciones de cromatina (*Kim 2005; Chen 2009*).

I.3.1 Biogénesis y modo de acción de los miRNAs en plantas

Los miRNAs se definen como RNAs de cadena sencilla con una longitud de 21-24nt. Son evolutivamente conservados y han sido identificados como reguladores centrales de la expresión génica en plantas. Los miRNAs de plantas controlan a nivel post-transcripcional procesos como la respuesta al estrés, la regulación por fitohormas, el desarrollo y la morfogénesis de distintos órganos de la planta (*Rogers y Chen 2013*).

La biogénesis de los miRNAs (Fig. 5) se lleva cabo tanto en el núcleo como en el citoplasma. Los genes que codifican para los miRNAs (genes MIR) son transcritos por la RNA polimerasa II originando transcritos primarios largos denominados pri-miRNAs (*Voinnet 2009*). Los pri-miRNAs, poseen una estructura muy particular de tallo y asa que posteriormente permite el reconocimiento por la enzima DICER-like 1 (DCL1, una ribonucleasa III-like) que interaccionan con la proteína DAWDLE (DDL) en su extremo 3' (*Yu et al., 2008*). Este complejo pri-miRNA-DDL interacciona con otro complejo proteico formado por DCL1, Serrate (SER) y HYL1. Este complejo proteico corta al pri-miRNA dando lugar al precursor de miRNA (pre-miRNA), el cual posee una longitud variable y menor que la del pri-miRNA, y mantiene la estructura de tallo y asa (*Jones-Rhoades 2012; Chen, 2009*). Posteriormente, un segundo corte por DCL1 en la región del asa da como resultado un RNA pequeño de doble cadena (18-24 nt) el cual es conocido como miRNA/miRNA*. El miRNA/miRNA* es metilado por HEN1 (proteína que contiene un dominio de unión a RNA de doble cadena y un dominio de metiltransferasa) y exportado al citoplasma por la proteína HASTY donde deben ser incorporados en las proteínas ARGONAUTE (AGO1). Para llevar a cabo su función regulatoria, se comienza con la selección de la cadena de RNA que va a actuar en la identificación del gen blanco y la eliminación de la cadena complementaria denominada miRNA* el cual es degradado por la RNAsa SDN (Ramachandra y Chen, 2008). El complejo miRNA-AGO1 se conoce como: complejo de silenciamiento inducido por RNA (RISC) (*Jones-Rhoades 2012*).

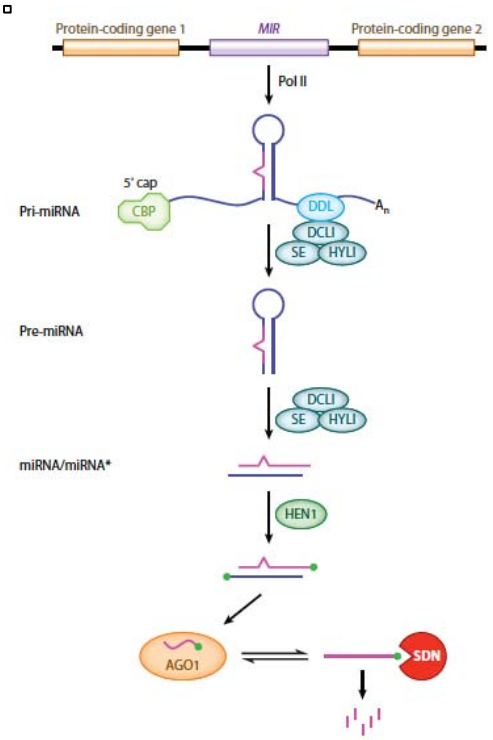


Figura 5. Biogénesis de los miRNAs. Los genes MIR son transcritos para dar lugar a un pri-miRNA, el cual posee las características de "cap" y poliA. El pri-miRNA es procesado en un pre-miRNA, el cual posteriormente originara el dúplex miRNA/miRNA* con la ayuda de las proteínas DICER-LIKE 1 (DCL1), SERRATE (SE) y HYPONASTIC LEAVES 1 (HYL1). Este dúplex es metilado por la enzima HEN1 y el miRNA maduro es incorporado al complejo AGO1 para llevar a cabo su función regulatoria (Tomado de *Chen 2009*).

Los miRNAs son reguladores negativos que pueden dirigir la acción de RISC para regular la expresión de sus genes blanco por medio de dos mecanismos: corte del RNA mensajero y represión traduccional (*Bartel 2004*). El corte del transcrito blanco se lleva a cabo por medio de la acción de las proteínas AGO, las cuales cortan al transcrito en el enlace fosfodiéster opuesto al nucleótido 10 u 11 de la cadena del miRNA en el dúplex miRNA-mRNA (Fig. 5).

Además los miRNAs pueden inhibir la traducción de su RNA mensajero blanco, en algunos casos se observa que el miRNA se une por complementariedad casi perfecta a su gen blanco pero los niveles de transcrito no disminuyen, mientras que los niveles de proteína si lo hacen, esto se conoce como represión traduccional, ya que el miRNA incorporado al complejo RISC se une a su gen blanco y no permite que el transcrito blanco se incorpore en los

ribosomas por lo que la traducción del gen blanco no se lleva a cabo (*Chen 2004; Aukermann y Sakai, 2004, Brodersen et al., 2008*).

Una característica de los miRNAs es que pueden poseer diferentes isoformas, esto se refiere a diferentes secuencias de un mismo miRNA maduro pero con uno o dos nucleótidos distintos en cualquiera de los dos extremos el 5' o el 3'. Las diferentes isoformas pueden provenir de precursores distintos o del mismo precursor que sea procesado de manera distinta (*Ebhardt et al., 2010*). Se ha observado que diferentes isoformas se expresan en diferentes partes de la planta por lo que pueden tener diferentes funciones aúnque pertenezcan a la misma familia (*Lelandais-Briere et al., 2009*).

Los genes blanco de miRNAs de plantas codifican esencialmente para factores de transcripción implicados en la regulación génica de funciones críticas. Por ejemplo, coordinan la morfogénesis de las hojas, la morfología de las flores, el número de órganos, las transiciones de desarrollo (de juvenil a reproductiva) y los cambios de fases (en la floración), la iniciación del desarrollo de raíz principal, entre otros. Además de controlar procesos de desarrollo, los miRNAs y blancos de plantas también poseen una función de respuestas tanto a estrés biótico (defensa contra patógenos e interacciones con simbiontes) como abiótico (respuesta a deficiencias nutricionales, entre otros) (*Vázquez 2009; Cartolano et al., 2007; Chen 2004; Yoshikawa et al., 2005; Kidner y Martienssen 2005*; *Formey et al., 2014*).

Actualmente, utilizando herramientas bioinformáticas, se puede predecir posibles genes blanco de un miRNA, analizando la complementariedad del mismo y del resto de los genes. Además también se pueden identificar posibles genes blanco de miRNAs analizando el degradoma. Esta es una técnica que se basa en que el corte inducido por el miRNA en el nucleótido 10 u 11 el cual produce fragmentos de mRNA poliadenilado en su extremo 3' y monofosfatado en su extremo 5'. Lo anterior es una firma para que por medio de secuenciación, se pueden identificar posibles genes blancos de miRNAs (Addo-Quaye et al., 2008).

Recientemente se ha descrito la relación de un miRNA con su gene blanco como nodo de un miRNA. Este concepto se refiere a la función que puede tener un miRNA y su gen blanco. Por ejemplo, un nodo puede ser específico (miR167c-ARF8) mientras que en otros casos el nodo puede ser más general e incluir a toda la familia de miRNAs y a toda una familia de genes blanco (miR319-TCP) (*Rubio-Somoza y Weigel 2011*).

I.3.2 Los miRNAs de leguminosas y su papel en la nodulación y la fijación simbiótica de nitrógeno (FSN)

Después del descubrimiento de los miRNAs, como reguladores globales de diferentes procesos, se han llevado a cabo diferentes estudios acerca del papel de los miRNAs en las leguminosas, incluyendo a la regulación de la FSN.

Debido al progreso en las técnicas de secuenciación, actualmente se ha facilitado la identificación de grandes familias de miRNAs y sus genes blanco en diferentes leguminosas. Por ejemplo, se encontraron, además de las familias de miRNAs conservadas, 229 familias nuevas para *Medicago truncatula*, 179 para soya (*Glycine max*) y 35 para *Lotus japonicus* (*Bustos-Sanmamed et al., 2013*).

Uno de los primeros trabajos reportados en donde se identifican miRNAs en leguminosas, es el descrito por *Subramanian et al.* (2008) en donde encontró que en raíces de soya inoculadas con *Bradyrhizobium japonicum,* expresa diferencialmente 20 familias de miRNAs que están conservadas en diferentes plantas tanto monocotiledóneas como dicotiledóneas, y 35 nuevas familias específicas de soya. Por otro lado, en *M. truncatula*, se identificaron 100 miRNAs específicos los cuales presentaron un patrón de expresión diferencial en nódulos y raíces en condiciones normales y de salinidad (*Lelandais-Brière et al., 2009*).

Recientemente, se han descrito familias conservadas y específicas de miRNAs en las leguminosas: soya, *M. truncatula*, *L. japonicus*, que se expresan diferencialmente en la organogénesis del nódulo (*Turner et al., 2012; Dong et al., 2013; de Luis et al., 2012*). *Formey*

et al. (2014) identificaron familias de miRNAs en raíces de *M. truncatula* crecidas bajo diferentes interacciones simbióticas y patogénicas así como también tratadas con factores Nod y factores Myc. En este trabajo, se reportan 52 nuevas familias de miRNAs y 60 nuevas variantes de las familias ya reportadas que se expresan diferencialmente en las raíces de *M. truncatula* tratadas tanto con NF como con factores Myc.

Actualmente existen tres miRNAs estudiados en procesos de nodulación en leguminosas. *Combier et al.* (2006) reportó la regulación espacial del miR169 y su factor de transcripción blanco MtNF-YA1 (antes conocido como MtHAP2-1) en nódulos de *M. truncatula*. El silenciamiento de MtNF-YA1 mediante RNAi resulto en un fenotipo alterado en el desarrollo de los nódulos los cuales no fueron capaces de fijar nitrógeno; algo similar sucedió en plantas que tenían sobreexpresado el miR169. El hecho que la expresión de MtNF-YA1 esté confinada a la zona meristemática del nódulo y que la expresión del miR169 se encuentre en la zona de infección sugiere que el miR169 sea el responsable de mantener la regulación espacial de MtHAP2-1 en el nódulo.

En contraste a lo reportado para miR169, la regulación espacial mediada por miR166 es diferente, debido a que tanto el miR166 como su gen blanco, el factor de transcripción HD-ZIP III, se co-expresan en las regiones vasculares y apicales de las raíces. Esta superposición de expresión puede estar relacionada con una regulación muy fina por parte del miR166 sobre su gen blanco. La función propuesta para esta regulación por parte de miR166 es mantener los niveles de HD-ZIP III en cierto nivel para tener una actividad meristemática adecuada; ya que se demostró que la sobreexpresión de miR166 afecta el patrón de haces vasculares en las raíces y también disminuye la formación de raíces laterales y nódulos (*Boualem et al.* 2008).

También se han caracterizado miRNAs específicos de soya que poseen un papel en la regulación de la nodulación. *Li et al.* (2010) observaron que la expresión de miR482 no fue afectada después de la inoculación con *Bradyrhizobium japonicum* en mutantes supernodulantes y no nodulantes de soya; mientras que la sobreexpresión de este miRNA en

raíces transgénicas provoca el aumento en el número de nódulos. El gen blanco de este miRNA son los genes R, los cuales están involucrados en la defensa contra patógenos, por lo que la expresión constitutiva de miR482 promueve la formación de nódulos al estar reprimiendo a genes de respuesta a patógenos.

También se ha descrito el papel de miR164, que tiene como gen blanco al factor de transcripción NAC1, en el desarrollo de los nódulos en *M. truncatula*. Se descubrió que la sobreexpresión de este miRNA afecta la organogénesis de los nódulos posiblemente a través de la desregulación de la respuesta a auxinas (*D'haeseleer et al., 2011*).

En *L. japonicus* se ha descrito el papel de miR171 y su gen blanco, el factor de transcripción perteneciente a la familia GRAS, NSP2 (*De Luis et al., 2012*). En *L. japonicus*, la isoforma c de miR171 posee una fuerte expresión en nódulos, la cual disminuye considerablemente cuando se compara con los nódulos no infectados producidos por una mutante de *Lotus*, la cual genera nódulos espontáneos en ausencia de *Mezorhizobium loti*. Los nódulos espontáneos de esta mutante y los nódulos silvestres no poseen diferencias morfológicas ni de desarrollo, lo cual sugiere que el aumento en la expresión de miR171c en nódulos efectivos, fijadores de nitrógeno, esta relacionado con la presencia del simbionte (*De Luis et al., 2012*).

Recientemente, *Turner et al.* (2013) describieron el papel de miR160 en nódulos de soya; este miRNA posee como genes blanco un conjunto de factores de respuesta a auxinas (ARF). La sobreexpresión de miR160 aumenta la sensibilidad a auxinas, lo cual provoca una disminución en la formación del primordio del nódulo aparentemente a través de una reducción en la sensibilidad a citocininas.

I.3.3 miRNAs de frijol

A pesar de la importancia agrícola del frijol existen pocos miRNAs (10 hasta la fecha) reportados en la base de datos de miRNAs (miRBase, www.mirbase.org).

Debido a esto nos pareció importante hacer una revisión sobre los miRNAs en la nodulación de frijol. Este artículo se publico en la revista: *The Journal of the International Legume Society* sobre los miRNAs en la nodulación del frijol el cual tiene como titulo: "MicroRNAs as post-transcriptional regulators in common bean (*Phaseolus vulgaris*)". El artículo se incluye en el Anexo 1.

Así como en otras plantas, en frijol también se han utilizando herramientas de clonación y secuenciación de RNAs pequeños para identificar familias de miRNAs tanto conservadas como específicas. Inicialmente *Arenas-Huertero et al.* (2009) identificaron 16 familias de miRNA conservadas y 8 familias específicas de frijol; además, demostraron la secuencia de los precursores de ciertos miRNAs y validaron los genes blanco de varios miRNAs como es el caso de miR156, miR164, miR172 entre otros.

Posteriormente, *Valdés-López et al.* (2010) realizaron un perfil de miRNAs en diferentes tejidos de frijol, utilizando la metodología de hibridación de macroarreglos de miRNAs, y observaron una expresión diferencial de diferentes miRNAs bajo condiciones de estrés abiótico, principalmente el estrés nutricional, como la deficiencia de fósforo, nitrógeno, hierro, toxicidad por manganeso y el pH ácido. Observaron que de los 32 diferentes miRNAs expresado en hojas, raíces y nódulos de frijol, 25 mostraron una expresión diferencial en los tejidos evaluados y bajo estrés abiótico, 16 fueron expresados en todas las condiciones evaluadas. Entre esos miRNAs que presentaban una expresión diferencial se encontró miR172, el cual es el miRNA analizado en el presente trabajo.

Con los avances en las metodologías de secuenciación masiva, se analizaron librerías de RNAs pequeños de diferentes tejidos como: raíces, semillas germinadas, botones florales (*Peláez et al., 2012*). En este trabajo, se identificaron 109 miRNAs pertenecientes a 29 familias conservadas y se predijeron 29 nuevos candidatos de miRNAs.

El genoma de frijol fue secuenciado recientemente (*Schmutz et al., 2014*) así que se puede acceder a esta secuencia genómica a través de Phytozome database (*Phaseolus*

*vulgaris*v 1.0, DOE-JGI and USDA-NIFA, http:://www.phytozome.net/commonbean). Esta herramienta y las librerías generadas por *Peláez et al.* (2012) han servido para mapear genes que codifican para miRNAs (genes MIR) y también para predecir las estructuras secundarias, de tallo-asa de sus precursores. Recientemente, utilizando el genoma de frijol recientemente liberado (*Schmutz et al., 2014*), 5 librerías de RNAs pequeños, obtenidas de 5 diferentes órganos de la planta (Peláez et al., 2012) y las secuencias del degradoma de frijol; se identificaron 185 miRNAs maduros, codificados en 307 precursores distribuidos en 98 familias y se identificaron 181 genes blanco (*Formey et al., 2015,* enviado a *BMC Genomics*).

En años recientes, se ha definido el papel de ciertos miRNAs en raíces y nódulos de frijol. *Valdés-López et al.* (2008) demostraron el papel de miR399 en las raíces de frijol bajo deficiencia de fosfato; este miRNA tiene como gen blanco a una ubiquitina E2 conjugasa, PHO2, la cual se encarga de inducir la degradación de proteínas de respuesta a fósforo, como son los transportadores de alta afinidad por fósforo en condiciones normales. En este trabajo se demostró que en raíces de frijol en condiciones de estrés por deficiencia de fósforo el miR399 se expresa y degrada su gen blanco (PHO2) para permitir que los genes de respuesta a fósforo actúen y la planta pueda contender contra el estrés, incrementando el contenido de fósforo interno. Además, se demostró que el factor de transcripción PvPHR1 (perteneciente a la familia de factores de transcripción MYB) actúa como el regulador positivo de miR399 induciendo su expresión en condiciones de deficiencia de fosfato. Por último, la acción de este miRNA es modulada a través de un RNA que no codifica para proteína, Pv4, en un proceso llamado mimetismo en condiciones de suficiencia de fósforo (*Franco-Zorrila et al., 2007*).

Recientemente, *Ramírez et al.* (2013) reportaron el papel del miR399 en dos genotipos de frijol con respuestas contrarias a la deficiencia de fósforo: DOR364 (genotipo sensible a la deficiencia de fósforo) y BAT477 (genotipo tolerante a la deficiencia de fósforo). Se demostró que mientras el nivel de transcrito de los reguladores esenciales en la respuesta a la deficiencia de fósforo: PvPHR1, miR399 y Pv4 mostraron un aumento en la expresión en raíces deficientes

de fósforo en ambos genotipos; el regulador negativo PHO2, presento una disminución de su expresión en el genotipo resistente BAT477 pero no en el genotipo sensible DOR364. En los dos genotipos se encontraron 4 posibles sitios de unión de miR399 a PHO2, tres de los cuales fueron idénticos en secuencia entre ellos, mientras que uno de ellos mostro 3 nucleótidos de diferencia entre los genotipos DOR364 y BAT477. Las diferencias de nucleótidos resultan en una diferencia entre la complementariedad de bases y la estabilidad del duplex del miR399 con PHO2. Esta diferencia se observó más claramente en el genotipo sensible (DOR364) en donde el dúplex miR399-PHO2 es menos estable por lo que miR399 no actúa eficientemente sobre su gen blanco PHO2. Esto se interpreto como un aumento en la actividad de PHO2 sobre la degradación de proteínas de respuesta a fósforo en condiciones de estrés ocasionando así la sensibilidad de DOR364 a la deficiencia de fósforo.

I.4 Regulación post-transcripcional del miR172 en los procesos de desarrollo

I.4.1 Función conservada en plantas

El gen de miR172, *MIR172*, fue de los primeros genes MIR de plantas identificados en un perfil de RNAs pequeños que se realizó en Arabidopsis (*Park et al., 2002*). La secuencia madura de este miRNA regula la expresión a nivel post- transcripcional por corte del mensajero blanco y por represión de la traducción de un factor de transcripción específico de plantas llamado *APETALA2* (*AP2*) (*Park et al., 2002; Chen 2004*). Además de regular a *AP2*, también tiene como genes blanco a otros genes de la misma familia de factores de transcripción (AP2-like): *TARGET OF EAT1* (*TOE1*), *TOE2*, *TOE3*, *SCHLAFMUTZE* (*SMZ*), y *SCHNARCHZAPFEN* (*SNZ*) (*Aukerman y Sakai 2003; Schmid et al., 2003*) los cuales, junto con AP2, poseen en su secuencia codificante una región complementaria al miR172. Estos AP2-like han sido reportados también para especies como maíz, arroz y cebada (Fig. 5).

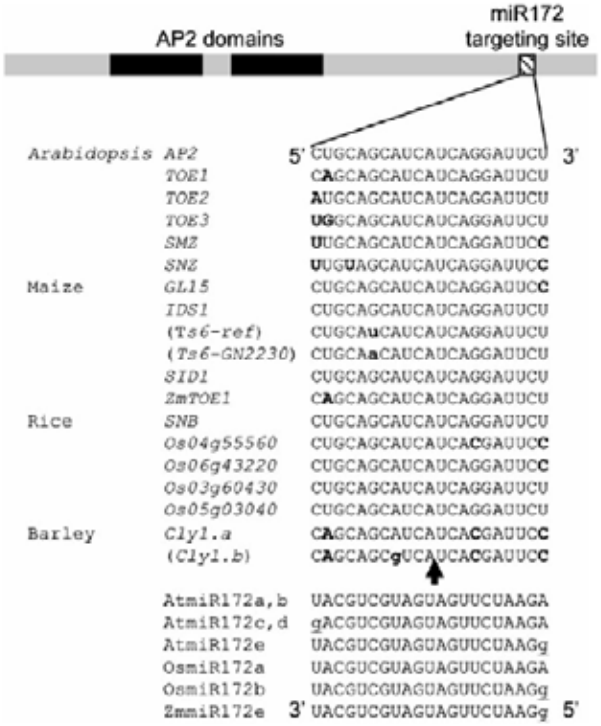


Figura 5. Sitios de unión de miR172 en AP2
(Tomado de *Zhu y Helliwell 2010*)

Este miRNA ha sido descubierto en una amplia variedad de especies tales como: helechos, gimnospermas, plantas con flores; aúnque no lo han descubierto en licopodios o musgos (*Axtell y Bartel 2005*).

La función principal del nodo miR172-AP2 ha sido ampliamente reportado en *Arabidopsis*. Se ha observado el fenotipo de plantas de *Arabidopsis* que sobreexpresen a miR172 y de plantas que tengan silenciado el gen AP2, lo cual genera un fenotipo similar: flores que pierden la identidad floral y la perdida de los pétalos (Chen 2004) (Fig. 6).

Figura 6. Fenotipo de la sobreexpresión de miR172 y silenciamiento de AP2 en flores de Arabidopsis. (A, WT; B, sobreexpresión de miR172 y C, silenciamiento de AP2. (Tomado de Chen 2004)

Posteriormente se analizaron los otros genes blanco de miR172, pertenecientes a la familia AP2, TOE1 y TOE2. Aukermann y Sakai et al. (2003) reportaron que el silenciamiento de TOE1 y TOE2 genera floración temprana al igual que la sobreexpresión de miR172 aúnque el fenotipo no es tan drástico como el silenciamiento de AP2, mientras que la sobreexpresión de TOE1 provoca una floración tardía, por lo que concluyen que los genes AP2-like son represores de la floración. La principal función de miR172 y AP2 es la identidad floral aúnque se sabe que también pueden estar involucrados en la transición de la fase vegetativa a la fase reproductiva que es donde comienza la floración (Zhu y Helliwell 2010).

Además de lo reportado en *Arabidopsis*, recientemente se ha reportado el papel de miR172/AP2 en maíz y arroz donde promueve la identidad floral en monocotiledóneas (*Lauter et al., 2005; Zhu et al., 2009*). Por lo anterior, miR172, es considerado un miRNA conservado.

El patrón de expresión de miR172 correlaciona inversamente con el del miR156 (*Wu et al., 2009*). Estudios en *Arabidopsis* han identificado que miR156 regula negativamente la expresión de miR172 de la siguiente manera: miR156 actúa rio arriba de miR172; este miRNA posee como gen blanco al factor de transcripción *SQUAMOSA PROMOTER BINDING PROTEIN LIKE9* (*SPL9*), el cual promueve la expresión de miR172 por medio de la activación directa de su transcripción (*Wu et al., 2009*).

En la regulación por parte de miR156 sobre miR172 (Figura 7) se observa que miR156 puede tener más de un SPL como gen blanco y los principales que actúan sobre miR172 de manera positiva son SPL9 y SPL10. Esta regulación positiva permitirá que miR172 regule negativamente a los genes blanco AP2 y AP2-like los cuales están involucrados en diferentes procesos de identidad floral y de transición de fase (Zhu y Helliwell 2010)

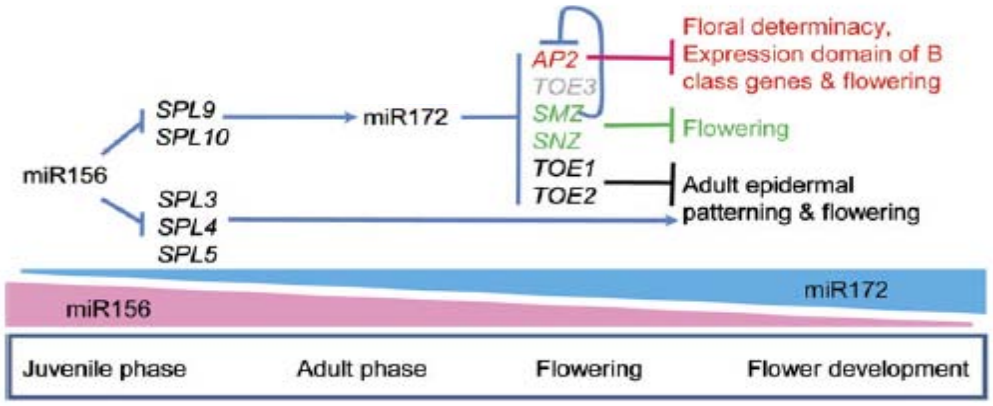


Figura 7. Modelo de regulación de miR156 y su gen blanco sobre miR172 y su gen blanco AP2 en el desarrollo floral de Arabidopsis. (Tomado de Zhu y Helliwell 2010)

En la mayoría de las plantas en donde se ha investigado la función de miR172, existe más de un gen de MIR172. Arabidopsis y maíz poseen 5 genes de MIR172; mientras que arroz solo posee 4. En Arabidopsis, existen 5 isoformas de miR172; la expresión de miR172a, b y c es elevada en plantas que alcanzan su estado reproductivo mientras que la expresión de miR172d y e es bastante baja y no altera el desarrollo de la planta (*Jung et al., 2007*). En arroz, la isoforma miR172c se expresa principalmente en plántulas pero no en granos en desarrollo (*Sunkar et al., 2008; Zhu et al., 2008*). Los diferentes trabajos antes mencionados demuestran que la expresión de las diferentes isoformas de este miRNA depende de los diferentes estadios de desarrollo de la planta y tipos de tejidos. Además, la expresión de miR172 también se ha visto afectada por la temperatura y los cambios de luz (*Jung et al., 2007; Lee et al., 2010*).

Por ultimo, tanto en monocotiledóneas como en dicotiledóneas, la expresión de miR172 se induce conforme la planta crece y se acumula en hojas y en botones florales (*Aukerman y Sakai 2003; Jung et al., 2007, Lauter et al., 2005*).

I.4.2 Función específica en leguminosas

Por otro lado, el conocimiento acerca de la función de este miRNA en leguminosas comienza a emerger. Se ha demostrado que su expresión en *M. truncatula* es más abundante en nódulos maduros con respecto a los niveles de expresión de este miRNA en raíces y que en

los nódulos maduros se expresa solamente en la zona de infección (*Lelandais-Brière et al., 2009*). También se ha demostrado que se induce su expresión en raíces sometidas a estrés salino.

En soya se evaluó la expresión de este miRNA en nódulos maduros (Wang et al., 2009) y en raíces inoculadas con *B. japonicum* (*Subramanian et al., 2008*). Se encontró que la abundancia de este miRNA aumenta desde 1 hora después de la inoculación con rhizobia así como en los nódulos maduros. Por otra parte, se demostró por medio del análisis de degradoma de genes blanco de miRNAs que el gen blanco de gma-miR172 es un factor de transcripción de la familia AP2 al igual que en Arabidpsis (*Song et al., 2011*).

En *Vigna unguiculuta* (cowpea) se identificaron diferentes familias de miRNAs conservados dentro de las cuales se identificó a miR172, principalmente la isoforma miR172b a la cual por medio de herramientas bioinformáticas se logró predecir tanto su estructura secundaria como su gen blanco el cual también pertenece a la familia de los AP2 (*Paul et al., 2011*).

Al mismo tiempo que se desarrollaba este proyecto de doctorado, se publicó un artículo (Yan et al., 2013), en donde se reporta por primera vez el papel de miR172 en la nodulación de soya. En este trabajo demuestran la inducción de miR172 en nódulos maduros de soya producidos por *B. japonicum*. La sobreexpresión de este miRNA en raíces transgénicas de soya incrementa el número de nódulos y la actividad de nitrogenasa. Por otro lado, los mismo autores demuestran la correlación negativa entre la expresión de miR156 y miR172 en nódulos de soya, por lo cual los autores proponen un modelo de regulación en el cual proponen a miR156 como regulador negativo de miR172 al igual que en Arabidopsis (Fig. 8). Finalmente, identificaron al gen blanco de miR172 de soya el cual es también un factor de transcripción de la familia AP2; la sobreexpresión de este gen provoca una disminución en la expresión de la hemoglobina no simbiótica por lo que los autores concluyen que el AP2 funciona como un

regulador negativo de la nodulación al reprimir la expresión de la hemoglobinas no simbióticas que se incrementan en los nódulos maduros y son importantes para la FSN (*Yan et al., 2013*).

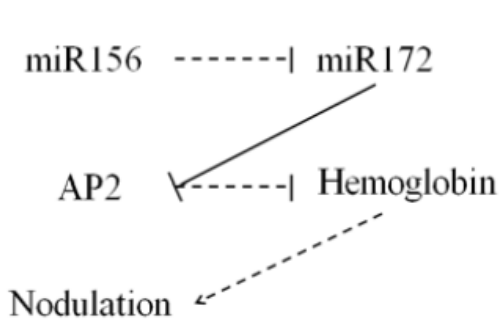


Figura 8. Modelo de regulación de miR172 y AP2 en nódulos de soya. Las líneas puntadeas significan regulación indirecta mientras que las líneas normales se refiere a una regulación directa. (Tomado de *Yan et al., 2013*)

Por ultimo, mientras el artículo de este trabajo doctoral estaba en revisión en la revista *Plant Physiology* se publicó otro (Wang et al., 2014) en donde se reporta la función de la isoforma miR172c en el proceso de nodulación de soya. Los autores demostraron que miR172c de soya es el encargado de modular tanto la infección por parte del rhizobia como la organogénesis del nódulo. Además, miR172c se induce en raíces de soya inoculadas con *B. japonicum* o tratadas con factores Nod y su expresión es altamente inducida durante el desarrollo del nódulo. Por otro lado, la sobreexpresión de este miRNA provoca cambios dramáticos en la iniciación del nódulo y en el número de los mismos. Finalmente, demostraron que miR172c regula la formación de nódulos a través de la represión de su gen blanco, que los autores denominaron: Nodule Number Control 1 (*NNC1*), el cual codifica para un factor de transcripción de la familia AP2. Demostraron que éste TF se une directamente al promotor de una nodulina temprana (*ENOD40*) para reprimir su expresión. Los autores proponen un modelo de regulación en donde, en presencia de rhizobia, los factores Nod que son percibidos por los receptores *NFR1/5* promueven la activación de miR172 y este, a su vez, reprime la expresión de su gen blanco *NNC1*, que permite la expresión de *ENOD40* para la iniciación de la formación del nódulo. Por el contrario, cuando no hay rhizobia, los niveles de *NNC1* son altos y mantienen reprimida la expresión de *ENOD40* y no hay formación de nódulos. Por ultimo, los

autores proponen que miR172c es reprimido por citocininas en el proceso de AON para prevenir una sobre-produccion de nódulos (Fig.9) (*Wang et al., 2014*). Sin embargo, no es claro si el miR172c controla respuestas tempranas que son críticas para establecer una simbiosis leguminosa-rhizobia efectiva.

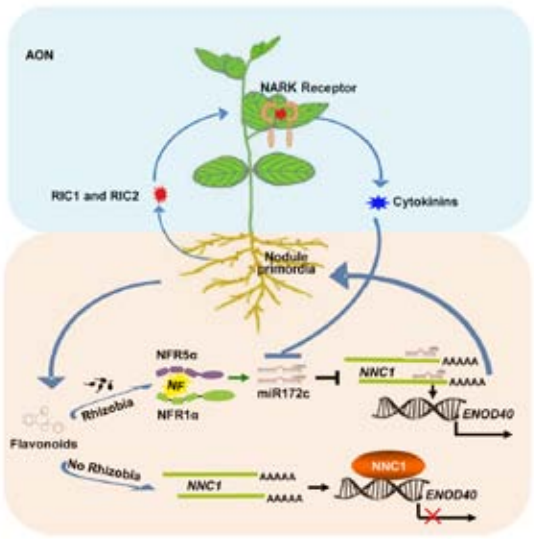


Figura 9. Modelo de regulación de miR172, AP2 y ENOD40 en la nodulación
(Tomado de *Wang et al., 2014*)

CAPITULO II OBJETIVOS

Como ya se planteo anteriormente, en México el frijol es muy importante para la dieta alimenticia del humano y la fijación simbiótica de nitrógeno es importante debido a los beneficios que otorga a los suelos con contenido de nitrógeno bajo. A pesar de la importancia del frijol, el conocimiento acerca de la participación de los miRNAs en el proceso de formación del nódulo y la fijación de nitrógeno es escaso por lo cual en este proyecto se plantearon los siguientes objetivos:

II.1 OBJETIVO GENERAL

Demostrar el papel regulador del nodo miR172-AP2 en la simbiosis de frijol-rhizobia.

II.1.1 OBJETIVOS PARTICULARES

- Analizar el patrón de expresión de las isoformas de miR172 y su genes blancos (*AP2*) en diferentes tejidos de frijol.
- Analizar la expresión de miR172c y su gen blanco *AP2-1* en simbiosis con *Rhizobium*, tanto en simbiosis efectiva como inefectiva.
- Demostrar la función de miR172/*AP2* a través del análisis del fenotipo simbiótico de plantas compuestas con alteraciones en la acción del nodo miR172c-*AP2-1*
- Identificar posibles genes blanco de la regulación transcripcional de *AP2-1* en la simbiosis frijol-*Rhizobium*.

CAPITULO III ANÁLISIS INICIALES DE miRNAs EN FRIJOL

III.1 miR398

Uno de los miRNAs que se ha trabajado en el laboratorio es el miR398 y yo participé en el proyecto del grupo sobre el análisis funcional de miR398 en frijol. De éste se derivó el artículo publicado por Naya et al. (2014) del cual soy co-autora y se presenta en el anexo I.

Se ha reportado el análisis del miR398 en plantas de Arabidopsis sometidas a estrés por deficiencia o por toxicidad a cobre (Cu). En condiciones de toxicidad por Cu se genera estrés oxidativo y aumenta la concentración de especies reactivas de oxígeno (ROS). Los ROS son generalmente moléculas pequeñas altamente reactivas que incluyen iones de oxígeno, radicales libres y peróxidos. Los ROS afectan funciones celulares dañando los ácidos nucleicos, oxidando a las proteínas y causando peroxidación de lípidos (Apel y Hirt 2004). Las superóxido dismutasas (SODs) constituyen la primer línea de defensa contra el estrés oxidativo producido por ROS, ya que convierten el superóxido en peróxido de hidrogeno (H_2O_2) y oxígeno molecular. De acuerdo al co-factor de metal que utilizan, las SODs son clasificadas en tres grupos: hierro SOD (FeSOD), manganeso SOD (MgSOD), y la de cobre-zinc SOD (Cu/ZnSOD, CSD), estas últimas han sido identificadas como genes blanco de miR398 (Sunkar et al., 2006).

En condiciones de toxicidad por Cu, se ha observado una disminución del miR398 lo que permite la acumulación del mRNA mensajero de CSDs para detoxificar las células y contender contra el estrés (Sunkar et al., 2006).

Cuando existe deficiencia de Cu en la planta, se incrementa la expresión del miR398 y se induce la degradación del mRNA de CSDs blanco. De esta manera el poco Cu disponible no se incorpora a las CSDs, sino que éste puede ser utilizado por otras proteínas que necesitan del cobre. Cuando es poco el cobre en la planta, las FeSOD se encargan de sustituir la función de las Cu/Zn SOD (Yamakasi et al., 2007).

En frijol se determinó la expresión de la isoforma b de miR398 por medio de Northern Blot y de su gen blanco conservado: Cu/Zn Superóxido Dismutasa (CSD1) por medio de PCR en tiempo real (qRT-PCR). En la Fig. 10 se muestra la expresión de miR398 por medio de Northern blot y la expresión de los genes blanco por qRT-PCR en condiciones de deficiencia y de toxicidad por Cu. Se observa es un aumento en la expresión de miR398 en condiciones de deficiencia de Cu en los nódulos, raíces y hojas y una disminución de su gen blanco CSD1. Mientras que en toxicidad por Cu se observa una disminución de la expresión de miR398b y un aumento en el transcrito de CDS1.

Además, en éste trabajo también se identificó un nuevo gen blanco para el de frijol miR398b, la Nodulina 19 (Nod19). Este gen fue reportado en *M. truncatula* como una nodulina que se expresa en nódulos jóvenes y raíces pero su función aún no ha sido demostrada (Gamas et al., 1996). Este gen también ha sido identificado en diferentes plantas tanto dicotiledóneas como monocotiledóneas; en soya se propuso como gen blanco de miR398 por medio de un análisis de degradoma (Song et al., 2011); en Arabidopsis el ortólogo de Nod19 se induce en respuesta a estrés como sequia por lo que se propone como un gen que puede tener un papel importante en la regulación a estrés (Kimura et al., 2003). En adición a la respuesta a estrés, también se demostró que Nod19 se induce en vainas de chícharo en presencia de un elicitor de insectos (Doss 2005). En la Fig. 10 se muestra que Nod19, al igual que CSD1, presentó un aumento en la expresión en condiciones de toxicidad por Cu. Este comportamiento fue contrario cuando las plantas se encontraban de deficiencia de Cu. Estos datos indican que el Nod19 también puede tener una función en estrés por cobre en frijol (Naya et al., 2014).

Se ha reportado la regulación de miR398 durante interacciones de *Arabidopsis* con *Pseudomonas syringae* (Jagadeeswaran et al., 2009). Las interacciones simbióticas y patogénicas poseen características en común como la síntesis de fenilpropanoides, inducción de peroxidasas, cambios en la concentración de Ca^{+2} intracelular y la producción de ROS. Debido a que miR398 y sus genes blanco CDS1 y *Nod19* parecen responder a la generación

de ROS, se analizó su expresión tanto en raíces inoculadas de frijol con *Rhizobium* a tiempos tempranos como en hojas de frijol infectadas con el hongo *Sclerotinia scleortiorum*. Se observó una disminución en la expresión de miR398b aumentando así la expresión de sus genes blanco (CDS1 y *Nod19*). En este trabajo se demostró por primera vez que en frijol el miR398 responde a la patogénesis de un hongo necrotrófico y a la inoculación por rhizobia, lo cual puede estar relacionado con la producción de ROS en ambas interacciones bióticas (Naya et al., 2014).

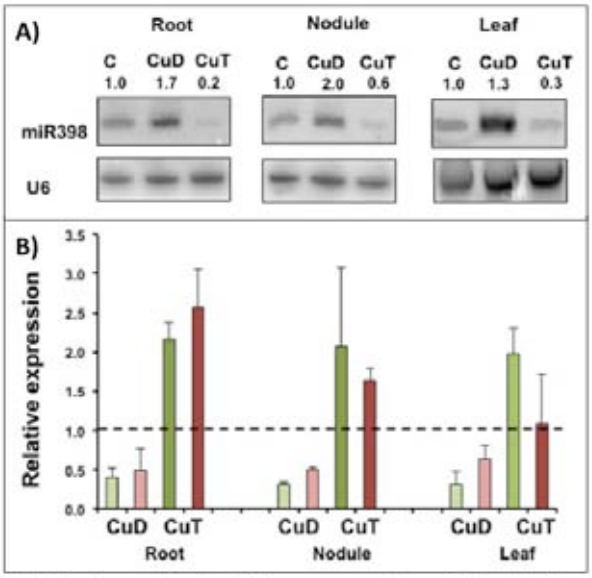


Figura 10. Patrón de expresión de miR38 y sus genes blanco CSD1 y Nod19 en diferentes tejidos de frijol bajo condiciones de deficiencia de cobre (CuD) y toxicidad por cobre (CuT). A, expresión de miR398 en raíces, nódulos y hojas de plantas crecidas bajo condiciones control (C) o en estrés (CuD o CuT) por Northern blot utilizando U6 snRNA como control de carga. La intensidad de la señal de las bandas de hibridación fueron calculadas por densitometría y se obtuvo el radio de expresión (estrés:control). B, expresión relativa de los genes blanco CDS1 (barras verdes) y Nod19 (barras rojas) en raíces, nódulos y hojas de plantas crecidas bajo condiciones de CuD (colores claros) y en estrés CuT (colores oscuros) calculada mediante qRT-PCR utilizando como gene constitutivo de referencia a UBC9. Los valores fueron normalizados al valor de la condición C, la cual fue establecida como 1 como se indica en la línea punteada. Los valores representan el promedio ± SD de tres replicas biológicas.

III.2 miR172

Este trabajo se enfoca en el análisis funcional de miR172 en frijol. Como ya se mencionó el miR172 es un regulador importante de procesos de desarrollo de la flor de Arabidopsis y de los nódulos de soya (Aukerman y Sakai 2003; Chen 2004; Yan et al., 2013; Wang et al., 2014).

En frijol, – por medio de la hibridación de macroarreglos de miRNAs – se detectó al miR172 solo en nódulos y no en hojas ni en raíces, los otros tejidos evaluados, por lo cual es el único miRNA que mostró un patrón de expresión "nódulo-específico ". Así mismo, también se observó que el miR172 de frijol se induce en deficiencias nutricionales como deficiencia de fosfato y de hierro así como en toxicidad por metales como manganeso (*Valdés-López et al., 2010*). Arenas-Huertero et al. (2009) validaron experimentalmente que el gen blanco de miR172 de frijol también es un factor de transcripción de la familia *AP2* (Phvul.005G138300) y que el miR172 induce el corte del mismo.

Por lo anterior, al inicio de este proyecto se evaluó la expresión de miR172 en diferentes condiciones de estrés nutricional para confirmar los resultados anteriores (Valdés-Lopez et al., 2010) y para proponer posibles funciones de este miRNA. Se evaluó su expresión por medio de Northern Blot en raíces, nódulos y hojas de frijol inoculadas con *R. etli*. Las plantas fueron crecidas en el sistema de hidroponía en condiciones óptimas (con solución nutritiva con todos los nutrientes excepto nitrógeno) (Franco & Munns, 1982) e inoculadas con *R. etli*. Después de 21 días, cuando las plantas ya formaron nódulos maduros, se transfirieron a condiciones de estrés por deficiencia de nutrientes utilizando solución nutritiva Franco & Munn carente de fósforo (Pd), fierro (Fed), potasio (Kd), azufre (Sd) y cobre (Cud). Las plantas se mantuvieron en estrés durante 7 días; después de ese tiempo los tejidos fueron colectados para el análisis de la expresión de miR172 (Fig. 11).

Los resultados de este análisis indican que el miR172 se induce claramente (3 – 4 veces) en nódulos sometidos a estrés por deficiencia de fósforo, azufre y cobre, mientras que

las hojas no responden a ningún estrés nutricional y en las raíces solo se induce su expresión en ausencia de P. Lo anterior confirmó lo reportado por Valdés-López et al. (2010) en cuanto a la inducción de miR172 en nódulos sometidos a estrés por deficiencia de fósforo.

Por medio de Northern blot y qRT-PCR, se observó que miR172 aumenta su expresión en nódulos maduros de frijol en comparación con otros tejidos y con nódulos jóvenes y senescentes. Estos resultados en conjunto, definieron el curso del proyecto y así, se continuó analizando el papel del miR172 en la organogénesis del nódulo dejando para trabajo futuro el análisis de su posible papel en condiciones de estrés nutricional.

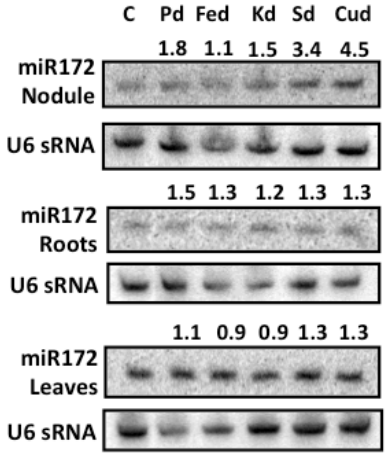


Figura 11. Expresión de miR172 por Northern Blot en plantas de frijol bajo condiciones de estrés nutricional. Los estreses evaluados fueron deficiencia de fosforo (Pd), deficiencia de fierro (Fed), deficiencia de potasio (Kd), deficiencia de azufre (Sd) y deficiencia de cobre (Cud). Se utilizó como control de carga el U6 snRNA. Los números indican la intensidad de la señal de las bandas de hibridación calculadas por medio de densitometría obteniendo el radio de expresión (estrés:control). La inducción significativa de la expresión se considero en valores <1.5.

Debido a que miR172 alcanza una mayor expresión en nódulos maduros efectivos de frijol, se planteó evaluar la expresión de miR172 en nódulos inefectivos, inducidos por un cepa de *Rhizobium etli* nod^{+}fix^{-}, *nifA*$^{-}$. Esta cepa de *R. etli* forma nódulos pero estos son incapaces de fijar nitrógeno debido a que posee una mutación en el regulador maestro de los genes *nif* : *nifA* (Girard et al., 2000). La expresión de miR172 (Fig. 12) disminuye considerablemente en los nódulos de frijol inefectivos mientras que la expresión de su gen blanco AP2 aumenta (Fig. 3, Capitulo IV). Con base en estos resultados, surgió la pregunta de si la disminución de la expresión de miR172 se debía a que en los nódulos inefectivos no se expresan los miRNAs o si

lo observado es propio y único de miR172 y pudiera estar relacionado con la función del mismo. Para responder a esta pregunta se analizo la expresión por medio de qRT-PCR de 4 miRNAs conservados: miR157, miR164, miR170 y miR396. Estos miRNAs se expresan en nódulos de frijol (Valdés-Lopez et al., 2010). Para miR164, miR170 y miR396 se demostró experimentalmente sus genes blanco los cuales son los factores de transcripción NAC, SCR y GRF-like respectivamente (Arenas-Huertero et al., 2009).

A pesar de que el nivel de expresión de los miRNAs analizados es diferente (Fig. 12), todos ellos muestran una expresión similar nódulos efectivos e inefectivos. De estos resultados se concluye que el miR172 es el único miRNA en el cual se ve afectada su expresión en nódulos inefectivos por lo que la función de miR172 pudiera estar relacionada con los nódulos funcionales.

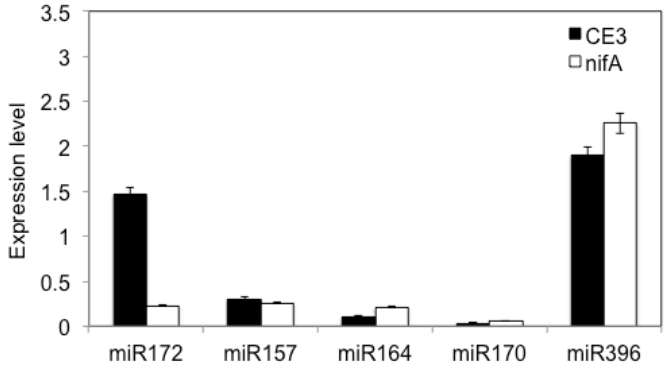


Figura 12. Expresión de miRNAs conservados por qRT-PCR en simbiosis efectiva e inefectiva. La expresión de los miRNAs conservados se analizó por medio de qRT-PCR utilizando como oligo 5' la secuencia de miRNA maduro. Para los cálculos de expresión se utilizó como miRNA constitutivo al miR159. Las plantas fueron crecidas en sistema de hidroponía e inoculadas con las dos cepas (*Rhizobium etli* y *Rhizobium etli nifA*⁻) por separado, después de 21 días se colectaron los nódulos y se analizó la expresión de los miRNAs. Los valores representan el promedio ± SD de tres replicas biológicas.

Como ya se mencionó anteriormente, miR172 es un miRNA conservado así como sus genes blanco que son factores de transcripción *AP2* (Fig.5) sin embargo, cabía la posibilidad de que en frijol el miR172 tuviera otros genes blanco. Como ya se mencionó el miR398 tiene sus dos genes conservados y, uno de ellos (Nod19), se regula en el estrés biótico y abiótico en frijol. Por esta razón se buscaron otros posibles genes blanco de miR172 no relacionados a la familia de factores de transcripción AP2.

Actualmente existe una herramienta bioinformática en donde se pueden predecir genes blanco de miRNAs con base en la complementariedad de bases del miRNA con los posibles genes blanco: (psRNAtarget: A plant small target analysis (http://plantgrn.noble.org/psRNATarget/ ; Dai and Zhao, 2011) y las bases e datos del Gene Index. Mediante esta herramienta y el Bean Gene Index (http://compbio.dfci.harvard.edu/tgi/) se predijeron varios genes blanco para miR172 que no pertenecían a la familia de *AP2* (Fig. 13). Estos posibles genes blanco estaban anotados como: S-receptor kinase-like protein, protein kinase CK2, phosphocholinecytidylytransferase CTP y allene oxidase cyclase.

El índice de complementariedad entre un miRNA y su gen blanco fue descrito por Jones-Rhoades y Bartel (2004). El criterio utilizado fue que el alineamiento abarque al menos 18 nucleótidos con un máximo de penalidad de 4.0. La calificación de penalidad se calcula considerando 0.5 puntos por cada G:U, un punto por cada "mismatch" diferente de G:U, y dos puntos por cada bucle de nucleótidos en cualquier cadena de RNA. En la Fig. 13 se muestran entre paréntesis la calificación de penalidad para cada gen blanco predicho para frijol. La calificación de penalidad que muestran los posibles genes blanco son altos debido a que normalmente una buena calificación de penalidad no es mayor que 1.0.

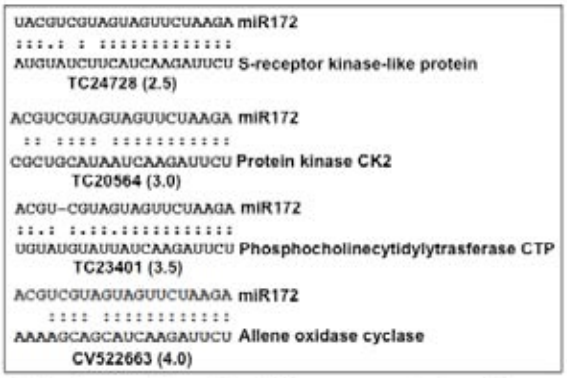


Figura 13. Predicción de genes blanco de miR172 no conservados. Se analizo por medio del sitio http://plantgrn.noble.org/psRNATarget/ los posibles genes blanco para miR172 utilizando el Gene Index de frijol. El límite de la calificación de penalidad se estableció en 4.0.

Después de predecir los otros posibles genes blanco bioinformáticamente, se analizó su expresión por qRT-PCR en nódulos y raíces de frijol utilizando oligonucleótidos específicos

para cada gen predicho. Los resultados se muestran en la Fig. 14. El nivel de miR172 es más abundante en nódulos que en raíces, mientras que el nivel de transcrito de AP2 aumenta en raíces de frijol y disminuye en los nódulos; esto se conoce como correlación negativa. La correlación negativa se observa en los miRNAs y sus genes blanco, ya que en los tejidos en donde se expresa el miRNA este induce la degradación del transcrito blanco y viceversa. La expresión de los otros genes blanco predichos (Fig. 14B) no presentó la correlación negativa con miR172 esperada, que si se observa con AP2. La mayoría de ellos se expresa en niveles similares o incluso en niveles mayores en nódulos. Por tanto, la interpretación de estos resultados es que los genes analizados no son genes blanco de miR172 de frijol y como en otras plantas, los genes blanco son solamente factores de transcripción de la familia AP2.

CAPITULO IV RESULTADOS

Nova-Franco B, Iñiguez LP, Valdés-López O, Alvarado-Affantranger X, Leíja A, Fuentes SI, Ramírez M, Paul S, Reyes JL, Girard L, Hernández G. (2015) The miR172c-AP2-1 Node as a Key Regulator of the Common Bean-Rhizobia Nitrogen Fixation Symbiosis. Plant Physiology, doi:http://dx.doi.org/10.1104/pp.114.255547.

The Micro-RNA72c-APETALA2-1 Node as a Key Regulator of the Common Bean-*Rhizobium etli* Nitrogen Fixation Symbiosis[1][OPEN]

Bárbara Nova-Franco, Luis P. Íñiguez, Oswaldo Valdés-López, Xochitl Alvarado-Affantranger, Alfonso Leija, Sara I. Fuentes, Mario Ramírez, Sujay Paul, José L. Reyes, Lourdes Girard, and Georgina Hernández*

Centro de Ciencias Genómicas (B.N.-F., L.P.I., A.L., S.I.F., M.R., S.P., L.G., G.H.), Laboratorio Nacional de Microscopía Avanzada (X.A.-A.), and Departamento de Biología Molecular de Plantas (J.L.R.), Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62210, Mexico; and Laboratorio de Genómica Funcional de Leguminosas, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlalnepantla, Edo de Mexico 54090, Mexico (O.V.-L.)

ORCID IDs: 0000-0002-2481-7715 (X.A.-A.); 0000-0001-5129-9741 (J.L.R.); 0000-0003-0004-9106 (L.G.); 0000-0001-6214-311X (G.H.).

Micro-RNAs are recognized as important posttranscriptional regulators in plants. The relevance of micro-RNAs as regulators of the legume-rhizobia nitrogen-fixing symbiosis is emerging. The objective of this work was to functionally characterize the role of micro-RNA172 (miR172) and its conserved target APETALA2 (AP2) transcription factor in the common bean (*Phaseolus vulgaris*)-*Rhizobium etli* symbiosis. Our expression analysis revealed that mature miR172c increased upon rhizobial infection and continued increasing during nodule development, reaching its maximum in mature nodules and decaying in senescent nodules. The expression of *AP2-1* target showed a negative correlation with miR172c expression. A drastic decrease in miR172c and high *AP2-1* mRNA levels were observed in ineffective nodules. Phenotypic analysis of composite bean plants with transgenic roots overexpressing miR172c or a mutated *AP2-1* insensitive to miR172c cleavage demonstrated the pivotal regulatory role of the miR172 node in the common bean-rhizobia symbiosis. Increased miR172 resulted in improved root growth, increased rhizobial infection, increased expression of early nodulation and autoregulation of nodulation genes, and improved nodulation and nitrogen fixation. In addition, these plants showed decreased sensitivity to nitrate inhibition of nodulation. Through transcriptome analysis, we identified 114 common bean genes that coexpressed with *AP2-1* and proposed these as being targets for transcriptional activation by *AP2-1*. Several of these genes are related to nodule senescence, and we propose that they have to be silenced, through miR172c-induced *AP2-1* cleavage, in active mature nodules. Our work sets the basis for exploring the miR172-mediated improvement of symbiotic nitrogen fixation in common bean, the most important grain legume for human consumption.

The symbiotic nitrogen fixation (SNF) occurring in the legume-rhizobia symbiosis takes place in root-developed specialized organs called nodules. Nodulation is a complex process that involves communication between rhizobia and legumes through molecular signals, including rhizobial lipochitin-oligosaccharide symbiotic signals known as nodulation factors (NFs), that triggers a root-signaling cascade essential for rhizobia infection (for review, see Crespi and Frugier, 2008; Oldroyd and Downie, 2008; Kouchi et al., 2010; Murray, 2011; Oldroyd, 2013). Nuclear Ca^{2+} oscillations, or calcium spiking, is one of the earliest NF-induced responses in legume root hairs. Perception and transduction of the calcium-spiking signal involves Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE (CCaMK), which interacts with the nuclear protein CYCLOPS, and other downstream components, such as the transcriptional regulators NODULATION SIGNALING PATHWAY (NSP1)/NSP2, NUCLEAR FACTOR YA1 (NF-YA1)/YA2, ETHYLENE-RESPONSIVE FACTOR REQUIRED FOR NODULATION1, and NODULE INCEPTION (NIN), which, in turn, control the expression of early nodulation genes.

Legumes strictly regulate the number of developing nodules in response to internal and external cues. An important internal cue is the systemic feedback regulatory mechanism called autoregulation of nodulation (AON), which consists of root-derived and shoot-derived long-distance signals. AON is initiated in response to rhizobial NF during nodule primordium formation by the root production of CLAVATA3/Embryo-Surrounding Region Protein-related (CLE) peptides (Reid et al., 2011a). Some CLE peptides are predicted, although not proven, to act as the ligand for a shoot CLAVATA1-like Leu-rich

[1] This work was supported by the Dirección General de Asuntos del Personal Académico/Universidad Nacional Autónoma de México (grant nos. PAPIIT: IN209710 and IN210814) and the Consejo Nacional de Ciencia y Tecnología, México (studentship nos. 351615 and 340334 to B.N.-F. and L.P.I., respectively).

* Address correspondence to gina@ccg.unam.mx.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Georgina Hernández (gina@ccg.unam.mx).

[OPEN] Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.114.255547

repeat receptor kinase (Okamoto et al., 2009). Activation of this receptor is proposed to initiate the production of a shoot-derived inhibitor that is transported to the root, where it inhibits further nodule formation (for review, see Magori and Kawaguchi, 2009; Ferguson et al., 2010; Kouchi et al., 2010; Reid et al., 2011b). Soil nitrogen is an important external cue for the control of nodulation (Streeter and Wong, 1988). Recent work indicates that nitrate inhibition of nodulation may function via an up-regulation of a nitrate-induced CLE peptide that is perceived by a Leu-rich repeat receptor kinase in the root (Okamoto et al., 2009; Reid et al., 2011a).

In recent years, microRNAs (miRNAs), a class of noncoding RNA 21 to 24 nucleotides in length, have been identified as central regulators of gene expression in plants, controlling fundamental processes such as stress response, phytohormone regulation, organ morphogenesis, and development (Rogers and Chen, 2013). The plant miRNA precursors, generally transcribed by RNA polymerase II, adopt stem-loop structures that are processed by several enzymes and generate mature miRNAs that are exported to the cytosol. The role of miRNAs in posttranscriptional regulation is mediated by the almost perfect complementarity with their target mRNAs, thereby causing their degradation or their translational inhibition (Zhang et al., 2006; Rogers and Chen, 2013).

Progress in high-throughput sequencing technologies has facilitated the genome-wide identification of large miRNA populations and their target mRNAs in different legumes (for review, see Simon et al., 2009; Bazin et al., 2012; Bustos-Sanmamed et al., 2013). Conserved and legume-specific miRNA families differentially expressed during nodule organogenesis have been reported for *Medicago truncatula*, soybean (*Glycine max*), and *Lotus japonicus* (Subramanian et al., 2008; Lelandais-Brière et al., 2009; De Luis et al., 2012; Turner et al., 2012; Dong et al., 2013). Recently, Formey et al. (2014) identified miRNAs from *M. truncatula* roots that respond to treatments with purified NF. However, evidence for the functional involvement of miRNAs in rhizobial infection and the functionality of nodules has only been obtained for a small number of candidates. The involvement of *M. truncatula* microRNA166 (miR166), miR169, and miR164 in nodule development has been reported. miR169 controls nodule meristem maintenance through the repression of *NF-YA1* (previously called *HAEM ACTIVATOR PROTEIN2-1*), a nodule-responsive transcription factor (TF; Combier et al., 2006), while miR166 and its target gene, *HOMEODOMAIN-LEUCINE ZIPPER protein of class III* TF, regulate meristem activity and vascular differentiation in roots and nodules (Boualem et al., 2008). The overexpression of miR164, a conserved miRNA targeting *NAC1* (for no apical meristem [NAM], Arabidopsis transcription activation factor [ATAF1-2], and cup-shaped cotyledon [CUC2] domain1) TF in roots, affected nodule organogenesis presumably through the deregulation of auxin responses (D'haeseleer et al., 2011). In soybean, the overexpression of miR482, miR1512, and miR1515 results in increased nodule numbers without affecting root development or the number of nodule primordia (Li et al., 2010). Recently, Turner et al. (2013) reported that the overexpression of soybean miR160, which targets a set of repressor auxin response factors, resulted in an enhanced sensitivity to auxin and inhibition of nodule development, apparently through a reduction in cytokinin sensitivity. Likewise, the overexpression of *M. truncatula* miR160 affected root gravitropism and nodule number (Bustos-Sanmamed et al., 2013). Specific variants of *L. japonicus* and *M. truncatula* miR171 target the GRAS-family *NSP2* TF, a key regulator of the common symbiotic pathway for rhizobial and arbuscular mychorrizal symbioses (Ariel et al., 2012; De Luis et al., 2012; Lauressergues at al., 2012). *M. truncatula* roots overexpressing miR171h showed deceased arbuscular mychorrizal colonization (Lauressergues at al., 2012), while in *L. japonicus*, miR171c regulates the maintenance and establishment of the nodule but not the bacterial infection (De Luis et al., 2012). In addition, the role of *L. japonicus* miR397 in nodule copper homeostasis, through the regulation of a member of the laccase copper protein family, has been documented (De Luis et al., 2012).

Common bean (*Phaseolus vulgaris*) is the most important crop legume for human consumption and the main source of proteins for people in African and Central/South American countries (Broughton et al., 2003). Our research is focused on identifying and functionally characterizing common bean miRNAs. High-throughput sequencing of small RNAs generated from different organs of common bean let us identify more than 100 conserved miRNAs and to predict novel miRNAs (Peláez et al., 2012). Common bean miRNAs that respond to drought, salinity, nutrient deficiencies, or metal toxicity stresses have been identified, and their target genes have been predicted or validated (Arenas-Huertero et al., 2009; Valdés-López et al., 2010; Contreras-Cubas et al., 2012). The roles of miR399 in the common bean root response to phosphorus deficiency (Valdés-López et al., 2008) and of miR398 in the regulation of copper homeostasis and response to biotic interactions (Naya et al., 2014) have been demonstrated. In this work, we analyzed the role of miR172 in common bean roots and nodules.

miR172 is conserved in all angiosperm lineages; its conserved targets are TFs from the *APETALA2* (*AP2*) family. The miR172 node that involves the miR156 node is one of the best-understood networks that regulate developmental timing in Arabidopsis (*Arabidopsis thaliana*) and other plants (Rubio-Somoza and Weigel, 2011). Aukerman and Sakai (2003) first described that miR172 promotes flowering by repressing *AP2* genes, primarily through translation inhibition (Chen, 2004) but also through mRNA cleavage (Kasschau et al., 2003; Jung et al., 2007). In addition, the miR172 node regulates the juvenile-to-adult phase transition during shoot development (Wu et al., 2009; Huijser and Schmid, 2011). Such developmental transitions are coordinated by the antagonistic activities of the miR156 and miR172 nodes. miR156 targets a subset of SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) TFs that bind to the miR712 promoter and directly promote its transcription, resulting in *AP2* silencing (Wu et al., 2009).

miR172 has been identified in the legumes *M. truncatula*, *L. japonicus*, soybean, and common bean; it is highly accumulated in mature nodules relative to other plant tissues (Lelandais-Brière et al., 2009; Wang et al., 2009; Valdes-López et al., 2010; De Luis et al., 2012). Yan et al. (2013) reported the regulation of soybean nodulation by miR172 that involves a complex regulatory circuit in which miR156 regulates miR172 expression, which, in turn, controls the level of its *AP2* target gene. They propose that *AP2*, directly or indirectly, controls the expression of nonsymbiotic hemoglobin, which is essential for regulating the levels of nodulation and nitrogenase activity (Yan et al., 2013). Very recently, Wang et al. (2014) demonstrated that soybean miR172c modulates rhizobial infection and nodule organogenesis. They showed that miR172c regulates nodule formation by repressing its target gene *NODULE NUMBER CONTROL1* (*NNC1*), an AP2 TF, which directly targets and represses the early nodulin gene *ENOD40* that plays a key role in nodulation. However, it is not clear whether miR172c controls early responses that are critical to establish a functional symbiosis between legumes and rhizobia.

The aim of this work was to analyze the role of the miR172 node in the common bean-rhizobia symbiosis. We determined an increased expression of miR172c upon rhizobial infection and during nodulation, showing a negative correlation with *AP2-1* expression. We achieved the overexpression of miR172c and of a mutagenized *AP2-1* insensitive to miR172 cleavage in composite common bean plants. Common bean plants with increased miR172c levels showed an improved symbiotic phenotype as well as lower sensitivity to nitrate inhibition of nodulation. We explored the possible role of AP2-1 as a transcriptional activator and/or repressor. Candidate target genes for downstream transcriptional activation by AP2-1 were identified; these could be relevant in the nodule senescence process. Our work extends the knowledge of miR172 function in the nodulation of common bean, an agronomically important legume.

RESULTS

Common Bean miR172 Isoforms and Target Genes

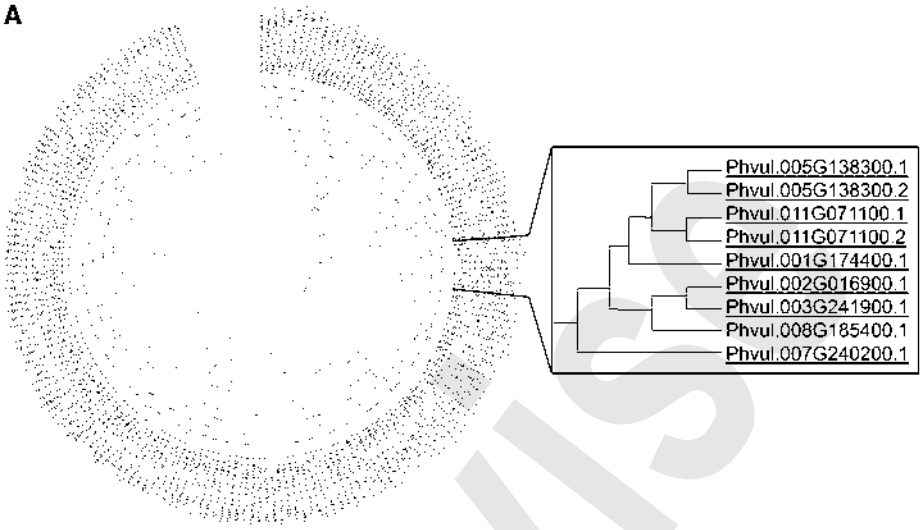
The Arabidopsis genome contains five loci that generate miR172 isoforms miR172a to miR172e, while 12 miR172 isoforms are reported for soybean (www.mirbase.org, version 20). The high-throughput small RNA sequencing analysis by Peláez et al. (2012) led us to identify four isoforms of common bean miR172.

In this work, we analyzed the recently published (Schmutz et al., 2014; www.phytozome.net/commonbean.php, v1.0) common bean genome sequence and identified six *MIR172* loci that map in different common bean chromosomes. The most stable secondary structure of the miR172 precursors was predicted, and these showed the expected stem-loop structure. The six isoforms of mature common bean miR172, 20 or 21 nucleotides long, were designated miR172a to miR172f (Supplemental Fig. S1). The nucleotide sequences of miR172a, miR172b, and miR172c isoforms differ. However, although encoded by different loci, the sequences of miR172e and miR172f are identical to miR172a, while miR172d is identical to miR172c (Supplemental Fig. S1).

The conserved targets for miR172 in different plants are transcripts that encode TFs from the *AP2* superfamily. In Arabidopsis, six *AP2* TF genes, *AP2*, *TARGET OF EARLY ACTIVATION TAGGED1* (*TOE1*), *TOE2*, *TOE3*, *SCHLAFMUTZE*, and *SCHNARCHZAPFEN*, which act as floral repressors, are targeted by miR172 and silenced through translation inhibition or cleavage (Aukerman and Sakai, 2003; Schmid et al., 2003; Chen, 2004; Jung et al., 2007). In soybean, 10 *AP2* TF genes have been proposed as miR172 targets (Song et al., 2011). A recent analysis of the common bean transcriptome by RNA sequencing (RNA-seq) combined with available gene calls (O'Rourke et al., 2014) identified 202 transcripts encoding AP2-type TFs. The phylogenetic tree generated using sequence alignments of the common bean AP2 proteins is depicted in Figure 1A. From the whole set (202), we identified eight *AP2* transcripts, encoded by six loci, with putative miR172 binding sites within their coding regions. The six predicted *AP2* target genes showed base pairing with the three different miR172 isoforms (Fig. 1B). In each case, the penalty score for miRNA:mRNA (Jones-Rhoades and Bartel, 2004) was low; the highest score was observed in Phvul.007G240200, with the three miR172 isoforms. From the predicted *AP2* targets, Phvul.005G138300, hereafter denominated as *AP2-1*, has been experimentally validated as a target of common bean miR172 (Arenas-Huertero et al., 2009). In addition, Phvul.011G071100 was identified as a target in a common bean degradome analysis (D. Formey, L.P. Íñiguez, P. Peláez, Y.F. Li, R. Sunkar, F. Sánchez, J.L. Reyes, and G. Hernández, unpublished data). Interestingly, the transcripts of *AP2* predicted targets were organized in a single clade of the phylogenetic tree (Fig. 1A). However, this clade also includes the Phvul.008G185400.1 transcript, which has an AP2 domain (http://www.phytozome.net/commonbean.php) but lacks a detectable miR172 binding site and, therefore, is not proposed as a target (Fig. 1A). The recently published *Phaseolus vulgaris* Gene Expression Atlas (*Pv* GEA; O'Rourke et al., 2014) showed a very low expression of this *AP2* gene in all the tissues reported (reads per kilobase per million = 6, highest values in leaves and pods). Therefore, Phvul.008G185400 *AP2* is perhaps highly expressed in tissue-, development-, or environment-specific conditions not yet analyzed and its transcript level could be regulated by factors other than miR172. In addition, we could not detect a Phvul.008G185400.1 ortholog in the soybean genome sequence, perhaps indicating that it could be a pseudogene.

Differential Expression of miR172, Predicted *AP2* Target Genes, miR156, and *SPL6* in Plant Tissues

The differential expression of miR172 isoforms in plant organs/tissues at different developmental stages has been reported for Arabidopsis and soybean (Aukerman



B

	miR172a	miR172b	miR172c
	miRNA:mRNA pairing (penalty score)		
Phvul.005G138300	5'-3' (0.5)	5'-3' (1.0)	5'-3' (1.0)
Phvul.011G071100	5'-3' (0.5)	5'-3' (1.0)	5'-3' (1.0)
Phvul.001G174400	5'-3' (1.5)	5'-3' (1.5)	5'-3' (0.5)
Phvul.002G016900	5'-3' (1.5)	5'-3' (1.5)	5'-3' (0.5)
Phvul.003G241900	5'-3' (1.5)	5'-3' (1.5)	5'-3' (0.5)
Phvul.007G240200	5'-3' (2.5)	5'-3' (2.5)	5'-3' (2.5)

Figure 1. Common bean *AP2* transcripts with predicted miR172 binding sites. A, Neighbor-joining tree of AP2 proteins retrieved from the common bean genome sequence (http://www.phytozome.net/commonbean.php, v1.0). The clade including *AP2* transcripts with miR172 binding sites (underlined) is shown in the inset. B, Pairing of the three different miR172 isoforms (a–c) with the predicted binding sites of the six different AP2 transcripts highlighted in A. Watson-Crick base pairing is indicated by lines, G:U base pairing is indicated by circles, and dashes indicate mismatches. Penalty scores, shown in parentheses, were calculated as described by Jones-Rhoades and Bartel (2004).

and Sakai, 2003; Wu et al., 2009; Yan et al., 2013; Wang et al., 2014). In this work, we performed expression analyses of the miR172 isoforms and their putative *AP2* target genes (Fig. 1) in different tissues of SNF common bean plants 18 d post inoculation (dpi) with *Rhizobium etli* (Fig. 2).

Northern-blot analysis revealed that mature miR172 transcripts were most abundant in nodules followed by flowers (Fig. 2A). The miR172a probe was used for blot hybridization, but the quantified signals might reflect the combined levels of miR172 isoforms whose sequences differ only in two nucleotides (Supplemental Fig. S1). For real-time quantitative reverse transcription (qRT)-PCR expression analysis, specific primers were synthesized for each of the miR172 isoforms (a, b, and c; Supplemental Fig. S1; Supplemental Table S1). The data obtained by northern-blot and qRT-PCR expression analyses showed similar trends regarding the highest levels in nodules and flowers and the lowest levels in roots and leaves (Fig. 2, A and B). The observed variation in the levels of cumulative miR172 expression for each tissue may be attributable to the different sensitivities of the two methods. In addition, qRT-PCR analysis revealed differential expression of the miR172 isoforms among tissues, especially in those tissues with higher cumulative levels. Nodules showed the highest level of miR172c and very low amounts of miR172a and miR172b, while flowers showed the highest level of miR172b, followed by miR172c and a low amount of miR172a (Fig. 2B).

The transcript levels of each of the *AP2* TF genes proposed as miR172 targets (Fig. 1) were determined by qRT-PCR (Fig. 2C) in tissues from SNF bean plants (Fig. 2C). Cumulative *AP2* transcript levels were very high in roots and very low in nodules, thus showing a negative correlation with cumulative miR172 levels in these tissues (Fig. 2, B and C). In roots, the most highly expressed of the *AP2* genes were Phvul.005G138300 (*AP2-1*) and Phvul.011G071100; *AP2-1* was also highly expressed in embryonic leaves (Fig. 2C). However, a distinct pattern was observed in flowers, where the expression of these two genes was negligible and Phvul.001G174400, Phvul.003G241900, and Phvul.002G16900 were highly expressed.

The miR156 node has been implicated in upstream negative regulation of the miR172 node (Rubio-Somoza and Weigel, 2011). Arabidopsis miR156 represses miR172 expression by targeting members of the *SPL* family of TFs that directly bind to the *MIR172* promoter and positively regulate its expression (Wu et al., 2009). Here,

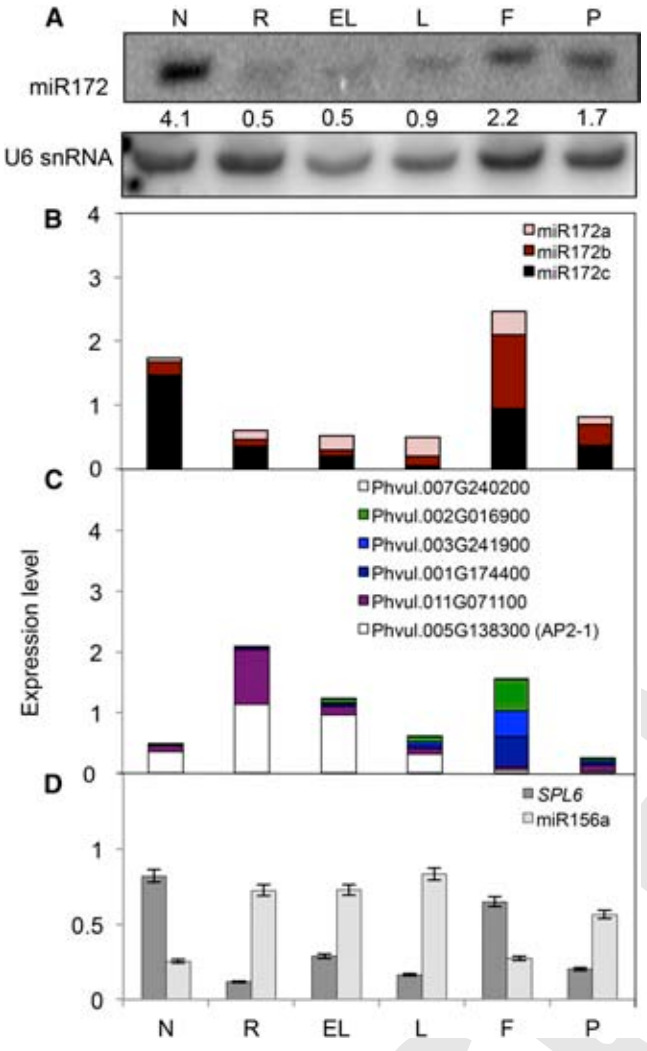


Figure 2. Expression analysis of miR172, *AP2* target genes, miR156a, and its *SPL* transcription factor target gene in different tissues of *R. etli* CE3-inoculated common bean plants (18 dpi). A, Mature miR172 levels detected by northern-blot analysis using U6 small nuclear RNA (snRNA) as a loading control for normalization. Signal intensities of the miR172 and U6 hybridization bands for each tissue were determined to calculate normalized expression levels. Numbers in each lane indicate normalized values of miR172 signal intensity. N, Nodules; R, roots; EL, embryonic leaves; L, leaves; F, flowers; P, pods. B to D, Transcript levels of mature miR172 isoforms (B; Supplemental Fig. S1), predicted *AP2* target genes (C; Fig. 1), and mature miR156a and its target gene *SPL6* (D) determined by qRT-PCR. Expression level refers to gene expression, based on threshold cycle (C_t) value, normalized with the expression of the housekeeping miR159 or *UBIQUITIN CONJUGATING ENZYME9* (*UBC9*) gene.

we determined the levels of mature miR156a (Peláez et al., 2012) in common bean tissues. The expression of miR156a was elevated in roots and leaves but low in nodules and flowers, showing an opposite trend of the cumulative expression of miR172 (Fig. 2D). We identified 32 *SPL* genes in the common bean genome, and 14 of these showed putative miR156 binding sites, including Phvul.009G165100, which was validated as a common bean miR156a target (Arenas-Huertero et al., 2009). Comparative sequence analysis with the Arabidopsis *SPL* gene family indicated that the common bean Phvul.009G165100 *SPL* gene is an ortholog to Arabidopsis *SPL6*. We analyzed the expression of the validated miR156a target *SPL6* gene in different common bean organs. As shown in Figure 2D, common bean *SPL6* was highly expressed in nodules and flowers while it was decreased in roots and leaves, thus showing a negative correlation with miR156 expression. We also searched for SPL transcription factor binding sites (TFBS) in the 5′ (promoter) region of each of the six *MIR172* loci mapped in the genome, but we could not identify any, while 35 other TFBS were present in one or more of these loci (Supplemental Table S2).

Our data (Fig. 2) showed miR172c as the isoform with the highest expression in nodules and low expression in roots. Its expression pattern is opposite that of *AP2-1* (Phvul.005G138300), the experimentally validated target (Arenas-Huertero et al., 2009) that showed the highest expression in roots. Therefore, we then focused our analysis on miR172c and *AP2-1* in common bean plants interacting with *R. etli*.

Expression Analysis of miR172 and *AP2-1* during Symbiosis

To assess a possible role of miR172c/*AP2-1* in the common bean-*R. etli* symbiosis, we determined their expression in inoculated roots (Fig. 3) and in effective nodules at different developmental stages (Fig. 4A).

To analyze miR172c/*AP2-1* regulation at early stages of the symbiosis, common bean plantlets were grown in plastic square bioassay dishes and *R. etli* inoculum was applied directly to the roots. For gene expression analysis, only the responsive root zone, where initial bacteria-host recognition takes place, was collected at the initial time (0 h) and at 3, 6, 12, 24, and 48 h post inoculation (hpi). The mature miR172c level increased significantly after 6 h, whereas the high expression level of the *AP2-1* target gene decreased significantly after 12 h; both transcript levels persisted until 48 h (Fig. 3A).

To investigate if miR172c up-regulation correlated with relevant events in the rhizobial infection process, we determined the expression of early nodulation genes in inoculated roots (Fig. 3, B–G). O'Rourke et al. (2014) identified common bean nodulation genes that were highly expressed in young and/or mature nodules and that are homologous to cognate nodulation genes previously identified in other legume species. From these, we selected six early nodulation genes for expression analysis: the TF genes *NF-YA1* (Phvul.001G196800.1), *NSP2* (Phvul.009G122700.1), and *NIN* (Phvul.009G115800); *CYCLOPS* (Phvul.002G128600.1), coding for a nuclear protein that interacts with CCaMK; *FLOTILLIN-LIKE2* (*FLOT2*; Phvul.009G090700.1), coding for a lipid raft component; and *ENOD40* (Phvul.008G291800), which lacks an open reading frame but encodes two small peptides and may function as a cell-cell signaling molecule for nodulation (Crespi and Frugier, 2008; Oldroyd and

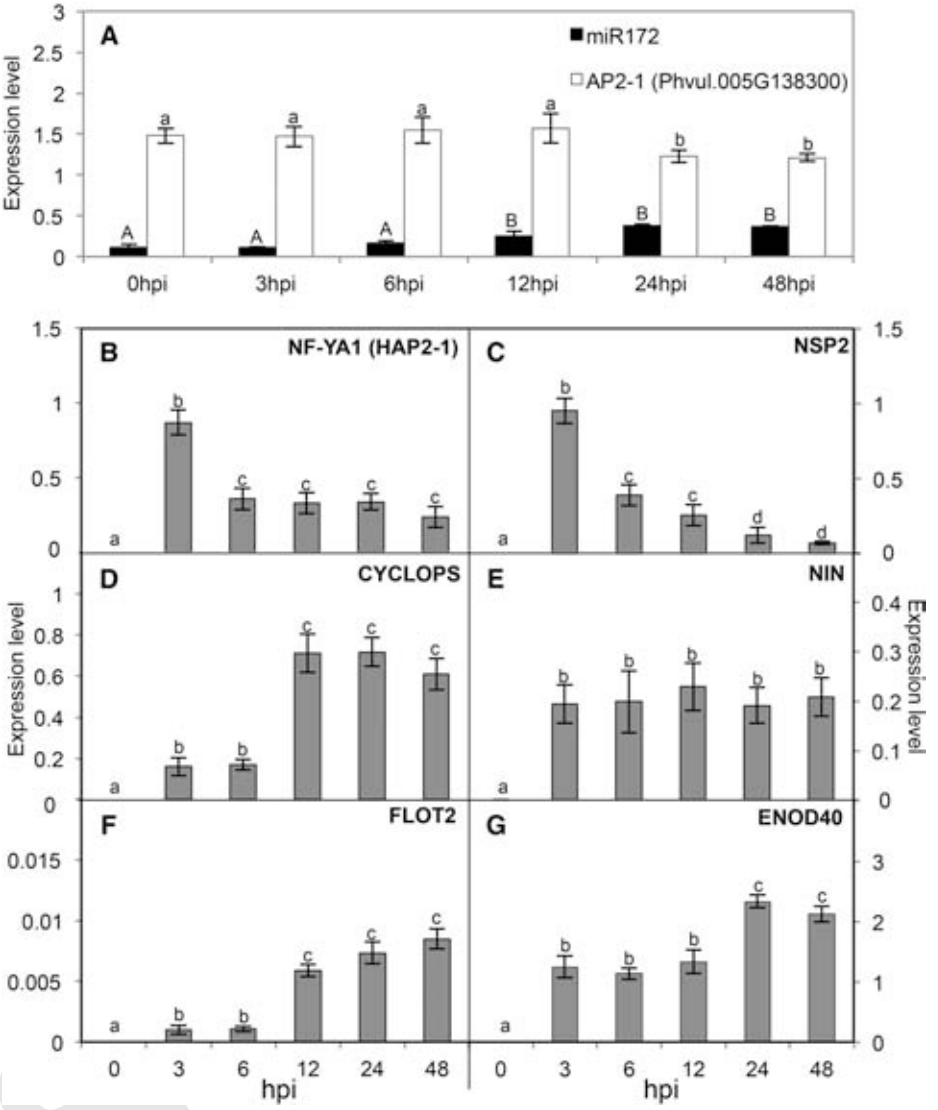


Figure 3. Increased expression of miR172c and of early nodulation genes upon rhizobial infection. Expression levels of mature miR172c, *AP2-1* (Phvul.005G138300; A), and early nodulation genes (B–G) were determined in roots inoculated with *R. etli* CE3 at the initial time (0) and after the indicated hpi. The common bean early nodulation genes were identified in the *Pv* GEA (O'Rourke et al., 2014): *NF-YA1*, Phvul.001G196800 (B); *NSP2*, Phvul.009G122700 (C); *CYCLOPS*, Phvul.002G128600 (D); *NIN*, Phvul.009G115800 (E); *FLOT2*, Phvul.009G090700 (F); and *ENOD40*, Phvul.002G064200 (G). Values represent averages ± SD from three biological replicates and two technical replicates each. Expression level refers to gene expression, based on C_t value, normalized with the expression of the housekeeping miR159 or *UBC9* gene. Different lowercase letters indicate statistically different groups (ANOVA, $P < 0.001$); in A, lowercase and uppercase letters were used for *AP2-1* and miR172c values, respectively.

Downie, 2008; Kouchi et al., 2010; Murray, 2011; Oldroyd, 2013). Figure 3, B to G, shows the expression levels of the early nodulation genes in the responsive zone of *R. etli*-inoculated roots. The expression of all the genes tested increased significantly after the initial time (3 h). Highest levels of *NF-YA1* and *NSP2* decreased gradually after 3 h. Increased *NIN* expression persisted, whereas *CYCLOPS* and *FLOT2* transcripts increased further after 6 h and persisted until 48 h. The *ENOD40* transcript level increased significantly after 12 h and persisted until 48 h.

We then analyzed the regulation of the miR172 node during the development of effective nodules elicited by the *R. etli* CE3 wild-type strain (Fig. 4A). Nodules from inoculated common bean plants were harvested at different developmental stages, as defined by the differential expression of nodule development marker genes (Ramírez et al., 2005; Van de Velde et al., 2006; Supplemental Table S3). Immature, prefixing, 13-dpi nodules showed the highest *ENOD55* expression and low (19.6%) nitrogenase activity. At 18 dpi, the nodules were fully developed and showed the highest nitrogenase activity and expression of the *PHOSPHOENOLPYRUVATE CARBOXYLASE* (*PEPc*) gene, essential for carbon assimilation in mature nodules (Ramírez et al., 2005). By 35 dpi, nodules had low nitrogenase activity (11%) and high *CYSTEINE PROTEINASE* (*CP*) gene expression, described as being specific for nodule senescence (Van de Velde et al., 2006; Supplemental Table S3). As shown in Figure 4A, the increased expression level of miR172c observed at 2 dpi (or 48 h in Fig. 3A) persisted in immature, prefixing nodules (13 dpi). In contrast, miR172c increased significantly to its highest level in mature, fully active nodules (18 dpi). Afterward, a drastic decrease in miR172c level was observed; it remained barely detectable until nodule senescence (35 dpi). Slightly decreased levels of *AP2-1* transcripts persisted in immature nodules (13 dpi), a further decrease was observed in mature 18-dpi nodules, and afterward, the level of *AP2-1* transcripts gradually increased. The lowest level of *AP2-1* correlated with the highest level of miR172c in mature nodules (18 dpi).

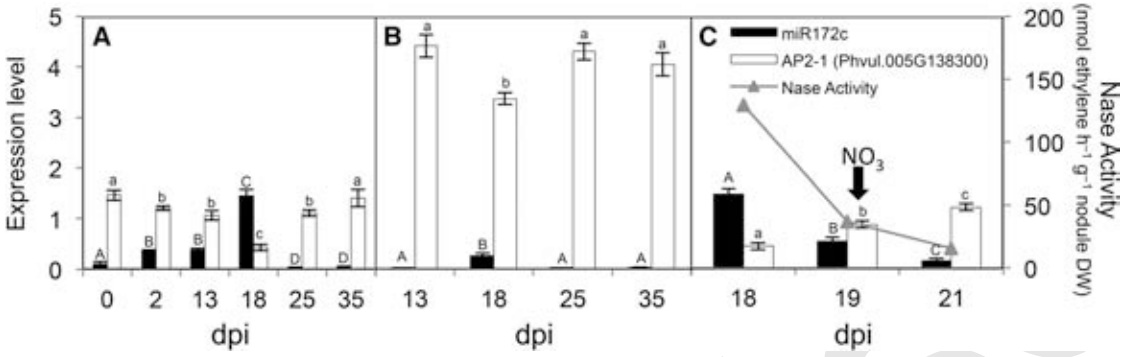


Figure 4. miR172c and *AP2-1* are differentially regulated in effective versus ineffective *R. etli* symbioses. Transcript levels were determined by qRT-PCR in inoculated roots or nodules harvested at the indicated dpi. A, Plants inoculated with the CE3 wild-type strain; determinations at 0 and 2 dpi were done in inoculated roots. B, Plants inoculated with the *fix*$^-$ *R. etli nifA*$^-$ mutant. C, Plants inoculated with the CE3 wild-type strain were grown for 18 d and watered with nitrogen-free nutrient solution. Subsequently, these plants were watered with nutrient solution supplemented with 10 mM KNO_3 (black arrow). Nitrogenase (Nase) activity and transcript levels were determined at 18 dpi and at 1 or 3 d after nitrate addition (19 or 21 dpi). Values represent averages ± SD from three biological replicates and two technical replicates each. Expression level refers to gene expression, based on C_t value, normalized with the expression of the housekeeping miR159 or *UBC9* gene. Different letters indicate statistically different groups (ANOVA, $P < 0.001$); lowercase and uppercase letters were used for *AP2-1* and miR172c values, respectively.

Taken together, these data suggest that miR172c is involved in rhizobial infection and nodule development/function.

Altered Expression of miR172c and *AP2-1* in Ineffective Symbioses

Because the maximum level of miR172c expression and *AP2-1* silencing correlated with the peak of SNF (Fig. 4A; Supplemental Table S2), we assessed the regulation of the miR172 node in ineffective, nonfixing common bean-*R. etli* symbioses.

The nitrogen fixation genes regulator A (NifA)/RNA polymerase sigma factor complex is a master regulator of the N_2 fixation genes in rhizobia. Transcriptional analysis of the *R. etli nifA*$^-$ (CFNX247) mutant strain demonstrated the *nifA* dependency of symbiotic genes on the symbiotic plasmid (Girard et al., 1996). The symbiotic phenotype of common bean plants inoculated with the *R. etli nifA*$^-$ mutant strain was drastically altered, as evidenced by a diminished amount of early-senescent nodules with few infected cells having bacteroids and devoid of nitrogenase activity and with symptoms characteristic of nitrogen deprivation in the leaves (Supplemental Fig. S2; Supplemental Table S3). As shown in Figure 4B, the ineffective nodules elicited by *R. etli nifA*$^-$ had nearly undetectable levels of miR172c, although a minor, but significant, increase was observed in 18-dpi ineffective nodules. Meanwhile, the *AP2-1* target gene was highly induced at the different developmental stages of ineffective nodules; these values were even higher (approximately 2-fold) than those observed during effective symbiosis (Fig. 4, A and B). A slight but significant decrease in *AP2-1* transcript was observed in 18-dpi ineffective nodules, when the miR172c level increased (Fig. 4B).

A similar effect was observed when the abolishment of SNF was achieved by adding nitrate to effective *R. etli*-elicited nodules (Fig. 4C), a well-known phenomenon in the legume-rhizobia symbiosis (Streeter and Wong, 1988). A short time (1 and 3 d) after nitrate addition, nitrogenase activity decreased drastically and nodules senesced (Fig. 4C; Supplemental Table S3). The latter correlated with the drastic decrease in mature miR172c and a concomitant increase of *AP2-1* transcript level in the ineffective nodules (Fig. 4C).

Taken together, these data indicate a contrasting regulation of miR172c/*AP2-1* expression in effective versus ineffective symbioses.

Effect of miR172c Overexpression on Root Development and Rhizobial SNF

To further investigate the role of miR172c and its target gene *AP2-1* in SNF, we aimed to modulate their expression in common bean composite plants with transgenic roots and untransformed aerial organs, generated through *Agrobacterium rhizogenes*-mediated genetic transformation. This protocol has been used as an alternative method for stable transformation in common bean and other recalcitrant species (Estrada-Navarrete et al., 2007). The construct for miR172 overexpression (OE172) contained the 35S cauliflower mosaic virus promoter fused to the miR172c precursor. The OEAP2m plasmid contained a mutagenized *AP2-1* gene that is insensitive to miR172 cleavage due to nucleotide substitutions in the miR172 binding site. Both constructs as well as the control empty vector (EV) contain the tdTomato (red fluorescent protein) reporter gene (Supplemental Fig. S3). We obtained several composite plants and determined the level of transgene expression for each plant (Supplemental Fig. S4). The OE172 composite plants showed very high levels of mature miR172c in both nodules and roots as well as a decreased level of *AP2-1* transcript. Roots and nodules of OEAP2m plants showed very high levels of *AP2-1*. The

variation in the degree of overexpression between individual transgenic roots is because each results from an independent transformation event.

We first assessed if miR172 overexpression affected the root phenotype of fertilized (noninoculated) common bean plants as compared with those inoculated with *R. etli*. As shown in Figure 5, roots with high miR172c showed increased biomass and density of secondary roots, both in fertilized and SNF composite plants. The opposite phenotype was observed in OEAP2m composite plants. These data indicate that miR172 had a positive effect on root biomass/architecture independent of the presence of rhizobia.

To analyze if the positive effect of miR172 on root development (Fig. 5) also affects rhizobial infection and SNF, we investigated the response of composite plants altered in miR172 content to *R. etli* infection, early symbiotic stages, and nodule development/function.

Figure 6 shows data for the analysis of rhizobial infection and early nodulation gene expression. For these experiments, the plastic square bioassay dish system was used for the inoculation and growth of OE172, EV, or OEAP2m composite plants. Notably, the amount of deformed root hairs was significantly higher in 48-hpi inoculated roots that overexpress miR172, while the opposite effect was observed in OEAP2m roots (Fig. 6A; Supplemental Fig. S5). A correlation of altered root hair deformation and the expression of early nodulation genes essential for rhizobial infection was observed after determining the transcript level of selected genes (*NF-YA1*, *NSP2*, *CYCLOPS*, *ENOD40*, *FLOT2*, and *NIN*) in the responsive root zone from 0 to 48 hpi (Fig. 6, B–G). All the genes tested showed increased expression in OE172 inoculated roots; *NIN* expression was increased only as compared with OEAP2m inoculated roots (Fig. 6, B–G).

Nodule number and nitrogenase activity as well as histological analysis of nodules stained with SYTO13 (a fluorescent dye binding nucleic acids) from composite plants overexpressing miR172 or *AP2m* and control (EV) plants are presented in Figures 7 and 8. At 14 and 21 dpi, OE172 plants showed increased nodule number and nitrogenase activity that correlated with higher *PEPc* and reduced *CP* expression (Fig. 7; Supplemental Table S4). In addition, OE172 plants showed accelerated nodule development: nodule primordia and well-formed nodules (approximately 50 per root) were easily observed at 5 and 7 dpi, respectively, while only some unorganized primordia and very few tiny nodules were seen in EV plants. In addition, young (7 and 14 dpi) nodules from OE172 plants were larger (increased perimeter; Fig. 8, A and B). In contrast, OEAP2m plants at 21 dpi had fewer nodules with diminished nitrogenase activity and higher *CP* expression (Fig. 7; Supplemental Table S4). At all time points analyzed, the OEAP2m nodules had significantly reduced perimeters (Fig. 8, A and B). Similar values for SYTO13 intensity per nodule area were obtained for EV, OE172, and OEAP2m nodules, indicating similar bacteroid densities in infected cells (Fig. 8C).

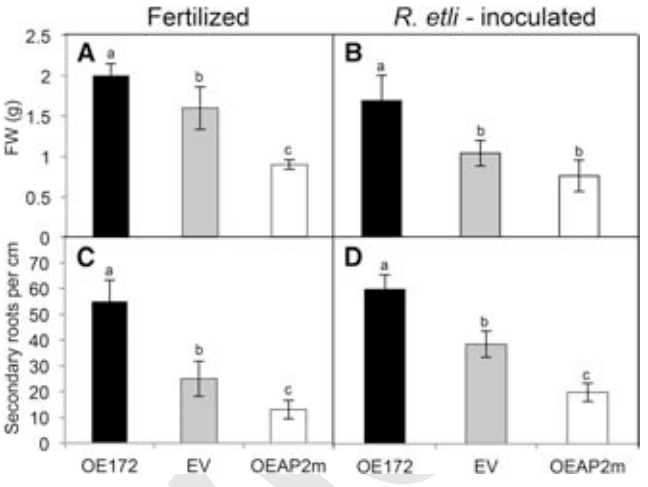


Figure 5. miR172c and *AP2-1* control the root development of fertilized and *R. etli*-inoculated common bean plants. Root fresh weight (FW; A and B) and density of secondary roots (C and D) were determined in composite bean plants with transgenic roots overexpressing miR172c (OE172) or a mutated insensitive *AP2-1* target gene (OEAP2m) as compared with control EV transformed roots. A set of composite plants was fertilized with full-nutrient solution for 10 d (A and C), and another set was inoculated with *R. etli* and watered with nitrogen-free nutrient solution for 21 dpi (B and D). Values represent averages ± SD from roots of eight independent composite plants each. Different lowercase letters indicate statistically different groups (ANOVA, $P < 0.001$).

We assessed if increased nodulation in OE172 common bean plants could be related to alterations in the AON. In soybean, AON involves long-distance signaling requiring the interaction of RHIZOBIA-INDUCED CLE peptides (RIC1/RIC2), with NODULE AUTOREGULATION RECEPTOR KINASE (NARK) in the leaf and the subsequent inhibition of nodulation via the production of a shoot-derived inhibitor. For local nitrate inhibition, the nitrate-induced CLE peptide (NIC1) interacts with NARK in the root, leading to a nitrate-induced inhibitor (Reid et al., 2011a). The homologous common bean *RIC* and *NIC* genes *RIC1* (Phvul.005G096900), *RIC2* (Phvul.011G135900), and *NIC1* (Phvul.005G097000) were identified from the *Pv* GEA (O'Rourke et al., 2014). As in soybean (Reid et al., 2011a), the common bean *RIC1* genes were expressed in inoculated common roots at early stages of rhizobial infection, while *RIC2* was expressed at later time points in prefixing and mature nodules. Figure 6, H and I, shows the expression levels of *RIC1* and *NIC1* genes in 48-hpi inoculated roots from OE172, EV, and OEAP2m plants. *RIC1* expression in control (EV) transgenic roots indicates the rhizobial induction of CLE-derived peptides for AON. Interestingly, the level of *RIC1* was decreased significantly in OE172 inoculated roots that showed increased nodulation, while it was increased significantly in OEAP2m roots with diminished nodulation (Fig. 6H). As expected, the expression of *NIC1* was low in the transgenic inoculated roots (Fig. 6I) under nitrogen-free conditions. These data point to the involvement of a common bean AON mechanism in the miR172 control of nodulation.

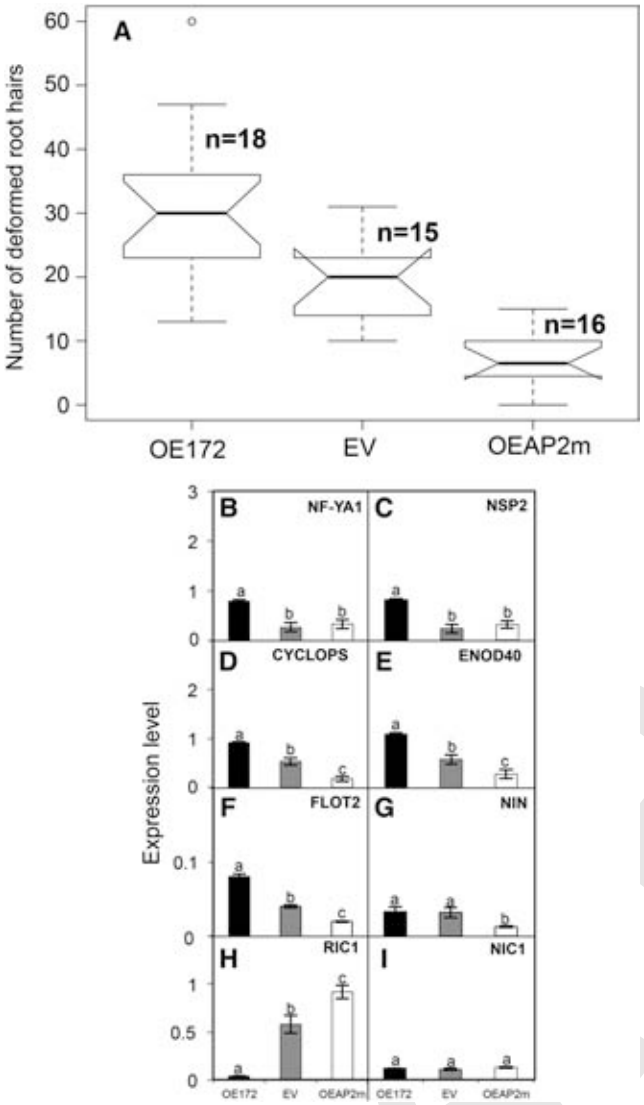


Figure 6. miR172c and *AP2-1* control the rhizobial infection of common bean roots. Roots from OE172, EV, or OEAP2m composite plants were inoculated with *R. etli* CE3, and at 48 hpi, the root responsive zones were harvested for analysis. A, Quantification of the number of deformed root hairs (branched and swollen root hair) per 0.5 cm; each box plot indicates the number of the transgenic roots analyzed for each construct. B to I, Expression analysis of selected early nodulation genes. Most gene identifiers are indicated in the legend to Figure 3; *RIC1*, Phvul.005G096900; and *NIC1*, Phvul005G097000. Values represent averages ± SD from three biological replicates and two technical replicates each. Expression level refers to gene expression, based on C_t value, normalized with the expression of the housekeeping *UBC9* gene. Different lowercase letters indicate statistically different groups (ANOVA, $P < 0.001$).

miR172c Overexpression Decreases the Sensitivity to Nitrate Inhibition of Rhizobial Symbiosis

Nitrogen (nitrate or ammonia) in the soil perceived by legume plants is an important external stimulus that inhibits nodulation as part of the AON mechanism. Considering the improved rhizobial infection and nodule development/function in common bean plants with increased miR172c (Figs. 6–8), we assessed if these alterations could be related to a decreased sensitivity to external nitrate inhibition of *R. etli* nodulation (Fig. 9). For this experiment, we applied a low nitrate concentration (1 mM) to *R. etli*-inoculated OE172, EV, and OEAP2m composite plants. The nodulation of inoculated OEAP2m plants, with low miR172c, was completely abolished when low nitrate was added. For this reason, we analyzed plants overexpressing miR172c as compared with control (EV) plants (Fig. 9). As expected for nitrate inhibition of the rhizobial infection process, the expression level of most early nodulation genes was reduced in EV-inoculated plants in the presence of nitrate (Fig. 9, A–F) as compared with the nitrogen-free condition (Fig. 6, B–G). Notably, in the presence of nitrate, the early nodulation genes *NF-YA1*, *NSP2*, *CYCLOPS*, *ENOD40*, and *FLOT2* showed significantly increased expression in OE172 inoculated roots (Fig. 9, A–F). In fact, the expression level of early nodulation genes in OE172 inoculated roots was similar in the absence (Fig. 6, B–G) or presence (Fig. 9, A–F) of nitrate. As expected, while the *RIC1* gene was barely detectable, *NIC1* was expressed in the responsive root zone of EV plants inoculated in the presence of nitrate, and it was increased in OE172 roots (Fig. 9, G–H).

Nitrate inhibition of nodulation was evident in control (EV) plants that presented delayed and diminished nodulation (Fig. 9I). These plants also showed decreased nitrogenase activity and *PEPc* expression and increased *ENOD55* (Fig. 9J; Supplemental Table S4) as compared with nitrogen-free inoculated plants (Fig. 7B). Notably, inoculated OE172 plants in the presence of nitrate showed few active nodules at 14 dpi, while at 21 dpi they had a similar number of mature nodules with slightly higher nitrogenase activity as compared with plants inoculated without nitrogen (Figs. 7 and 9, I and J; Supplemental Table S4). In addition, we observed that a higher nitrate concentration (3 mM) totally blocked the nodulation of EV plants, while OE172 plants were able to form active nodules (approximately 100 per root) at 21 dpi.

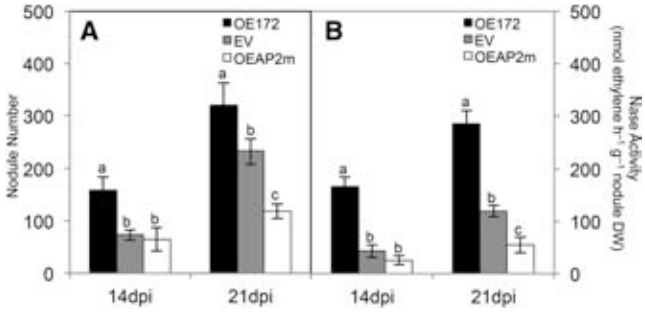


Figure 7. miR172c and *AP2-1* control nodule number (A) and nitrogenase (Nase) activity (B) of SNF common bean. Plants were inoculated with *R. etli* CE3 for the indicated dpi. Values represent averages ± SD from five replicate samples per time point. Different lowercase letters from each set of values at different dpi indicate statistically different groups (ANOVA, $P < 0.001$).

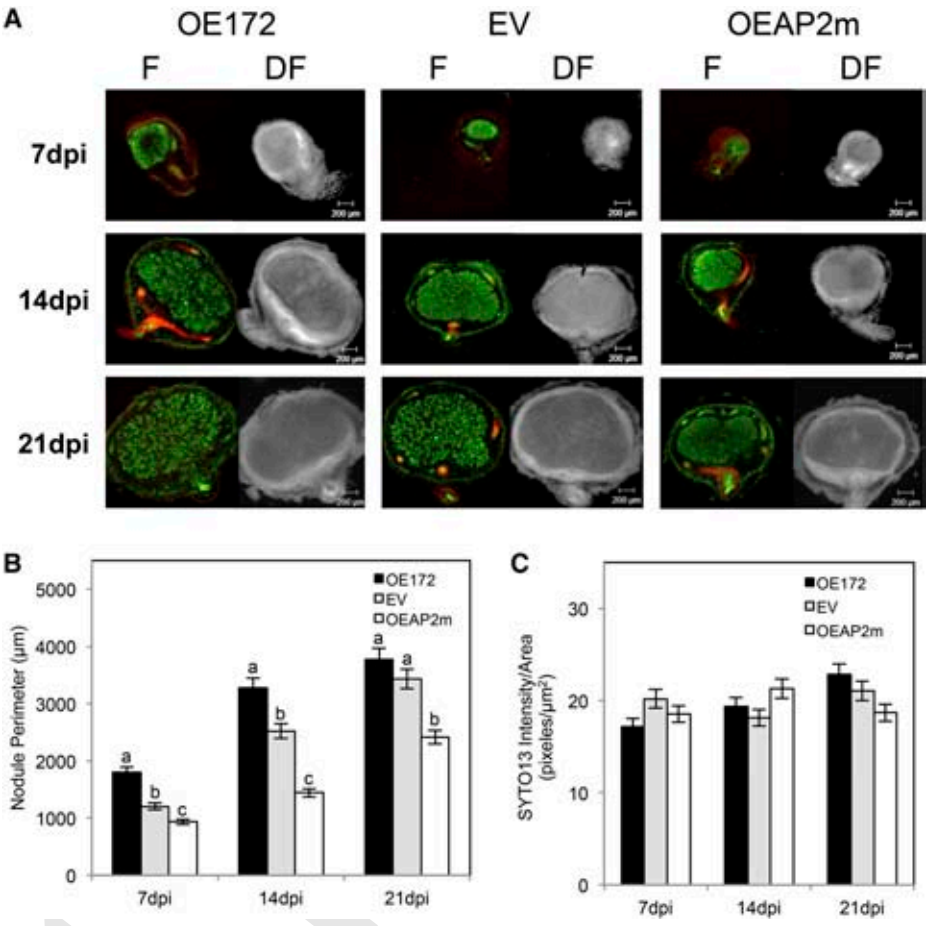


Figure 8. Alterations in the nodule development of OE172 and OEAP2m *R. etli*-inoculated composite bean plants. A, Fluorescent (F; left) and corresponding dark-field (DF; right) micrographs of central sections of nodules harvested at the indicated dpi. Red fluorescence from the tdTomato reporter gene expressed in *A. rhizogenes* transformed roots and green fluorescence from SYTO13 staining were observed. Magnification = 5×. B and C, Nodule perimeter (B) and SYTO13 fluorescence intensity per infection area (C) were calculated using the ImageJ program. Values represent averages ± SD from 10 replicate nodule images per condition. Different lowercase letters in B indicate statistically different groups (ANOVA, $P < 0.001$); values from C were not statistically different.

Exploring the Downstream *AP2-1* Regulation in SNF Plants

TFs from the *AP2* superfamily are widespread in plants and control diverse developmental programs and stress responses. Different *AP2* family members have been classified as activators or repressors of specific target genes (Licausi et al., 2013). In this work, we aimed to predict target genes for AP2-1 transcriptional activation or repression by analyzing data from the root and nodule libraries reported in the *Pv* GEA (O'Rourke et al., 2014), especially those from young roots and mature effective nodules that were derived from common bean tissues similar to those analyzed in this work. However, a caveat of this analysis is that the *Pv* GEA does not include libraries from roots inoculated with rhizobia for short periods, so we could not predict AP2-1 targets that would be regulated during the rhizobial infection process.

As shown in Figures 2 and 4, *AP2-1* showed high expression in common bean roots as opposed to mature nodules. We hypothesized that genes with an expression pattern similar to *AP2-1* are likely to be involved in root function/development and to be positively regulated by *AP2-1*, thus providing information on a possible mechanism of action of miR172/*AP2-1*. We identified 114 genes that had an expression pattern similar to *AP2-1*, designated *AP2-1* coexpressed genes (Supplemental Fig. S6). In order to support the latter contention, we searched for TFBS in the 5′ promoter region of *AP2-1* coexpressed genes. Besides WRKY, the most statistically overrepresented ($P = 0$ and 0.001) TFBS were ETHYLENE-RESPONSIVE FACTOR2 (ERF2) and DEHYDRATION-RESPONSIVE ELEMENT BINDING 1B (DREB1B), which belong to the AP2 superfamily. These TFBS were identified in 82% of *AP2-1* coexpressed genes (Supplemental Table S5).

Sixty-seven of the 114 *AP2-1* coexpressed genes could be assigned to a Gene Ontology (GO) category (Table I; Supplemental Table S5). The most statistically significant ($P = 0.006$) assigned GO category, which included 19 coexpressed genes, is GO:0004672, associated with protein kinase activity (Table I). We validated by qRT-PCR the expression of protein kinase activity genes in roots and nodules of control (EV) plants and also of plants overexpressing *AP2-1*. We hypothesized that those genes positively targeted by *AP2-1*, with high expression in roots and low expression in nodules from wild-type or control EV plants (Supplemental Fig. S6), would show a different expression pattern, higher and/or similar in roots and nodules, in OEAP2m plants that have constitutively enhanced expression of the *AP2-1* transcriptional regulator. Table II shows six *AP2-1* coexpressed genes assigned to the protein kinase activity category (GO:0004672) whose expression levels agree with our hypothesis. In control

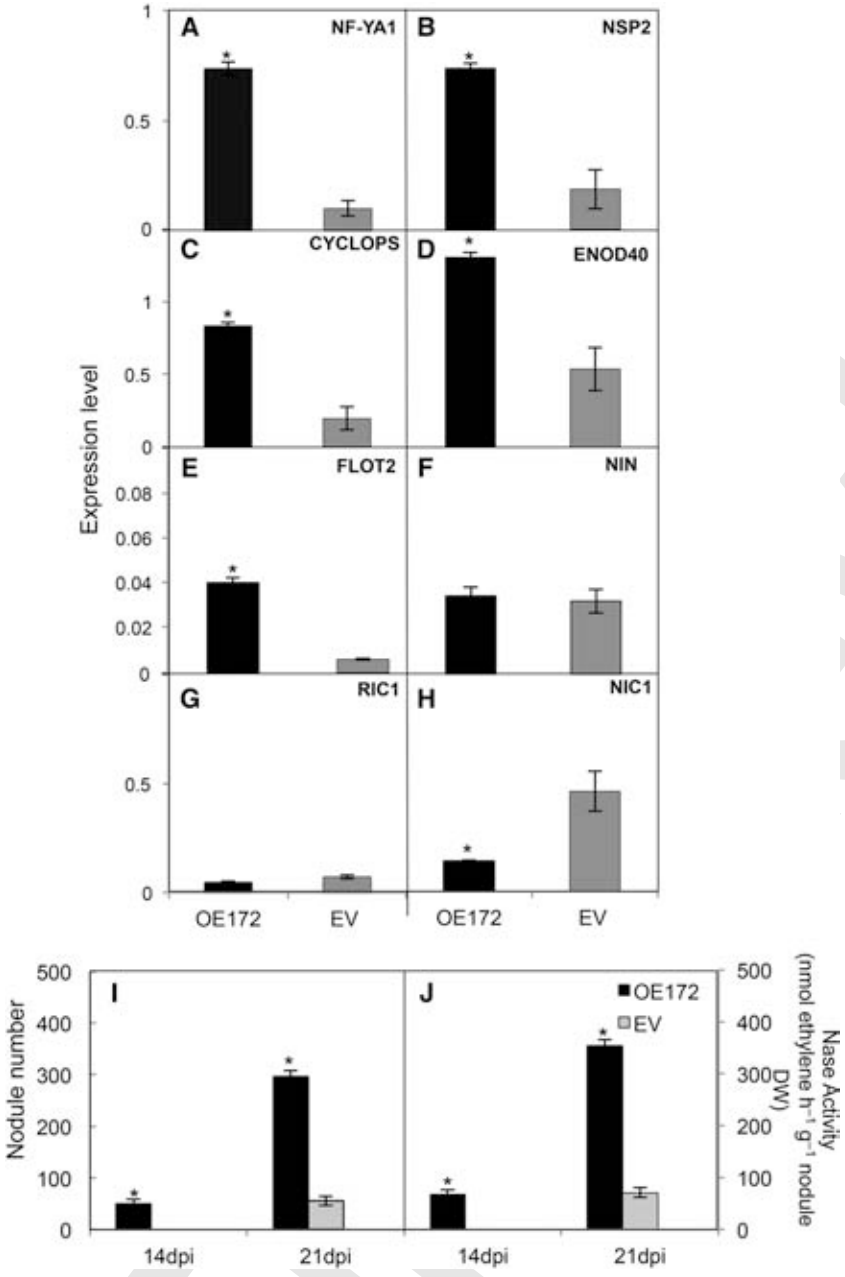


Figure 9. miR172c controls the sensitivity to nitrate inhibition of rhizobial symbiosis. To analyze the effect of nitrate in the symbiosis of *R. etli*-inoculated OE172 or EV composite plants, 1 mM KNO_3 was added to the nutrient solution used to water each set of plants daily. A to H, Expression analysis of selected early nodulation genes determined at 48 hpi in the root responsive zone. Gene identifiers are indicated in the legends to Figures 3 and 6. Values represent averages ± SD from three biological replicates and two technical replicates each. Expression level refers to gene expression, based on C_t value, normalized with the expression of the housekeeping *UBC9* gene. I and J, Nodules were counted (I) and nitrogenase (Nase) activity (J) was determined at the indicated dpi. Values represent averages ± SD from five replicate samples per time point. Asterisks indicate the level of statistically significant difference, if any, among values from OE172 and EV roots (Student's *t* test, $P < 0.01$).

(EV) plants, these genes were highly expressed in roots as compared with nodules, similar to *AP2-1*, thus validating the *Pv* GEA data (O'Rourke et al., 2014; Supplemental Fig. S6), while they showed an increased and/or similar expression in both tissues from OEAP2m plants (Table II). Interestingly, these genes shared the DREB, ERF, or both TFBS in their promoter regions (Table II; Supplemental Table S5). Transcriptomic analyses of *M. truncatula* nodule senescence have shown that protein kinases genes are one of the highly induced gene classes at different stages of this process (Van de Velde et al., 2006; Pérez Guerra et al., 2010). On this basis, we asked if the some of the *AP2-1* coexpressed genes from the protein kinase activity GO category might be related to nodule senescence in common bean. As shown in Table II, four of the protein kinase activity genes analyzed (Phvul.002G326600, Phvul.007G049500, Phvul.007G049000, and Phvul.006G174500) were induced in senescent (35 dpi) as compared with mature (18 dpi) nodules, thus indicating their possible relation to common bean nodule senescence.

The root/nodule expression pattern of *AP2-1* was opposite that of miR172c, which showed highest expression in mature effective nodules and very low expression in young roots (Figs. 2 and 4). O'Rourke et al. (2014) have described a set of 402 common bean genes highly expressed in mature effective nodules as compared with all other tissues analyzed in the *Pv* GEA. These genes are likely involved in the establishment of symbiosis and SNF as supported by their assigned GO categories (O'Rourke et al., 2014). Here, we hypothesized

Table I. *GO categories statistically overrepresented for genes coexpressed with AP2-1 in roots*

GO Identifier	Description (Molecular Function)	*P*
GO:0004672	Protein kinase activity	0.006
GO:0008703	5-Amino-6-(5-phosphoribosylamino)uracil reductase activity	0.016
GO:0005516	Calmodulin binding	0.018
GO:0005471	ATP:ADP antiporter activity	0.024
GO:0004674	Protein Ser/Thr kinase activity	0.036
GO:0004351	Glu decarboxylase activity	0.055
GO:0004435	Phosphatidylinositol phospholipase C activity	0.055

that these nodule-enhanced genes, lowly expressed in young roots with increased *AP2-1* (Figs. 2 and 4), are candidates for AP2-1 negative regulation. To this end, we searched for TFBS in the 5′ promoter region of nodule-enhanced genes, but we did not find a statistical overrepresentation of AP2 TFBS.

DISCUSSION

The key role of the miR172 node in Arabidopsis flowering time and phase transition is well known; similar roles have also been documented in maize (*Zea mays*), rice (*Oryza sativa*), and barley (*Hordeum vulgare*; Zhu and Helliwell, 2011). Besides conserved roles, specialized/particular species-specific functions of miR172, such as the induction of tuberization in potato (*Solanum tuberosum*), have been reported (Martin et al., 2009). In legumes, conserved roles of the miR172 node have been documented for *L. japonicus* (control of flowering time; Yamashino et al., 2013) and soybean (control of juvenile-to-adult phase transition; Yoshikawa et al., 2013). In addition, the control of nodulation during the rhizobia symbiosis has been proposed as a family-specific acquired function of miR172 in different legumes and has been demonstrated for soybean (Yan et al., 2013; Wang et al., 2014). In this work, we identified the miR172 node as a relevant regulator of rhizobial infection and nodulation in common bean.

We propose that different miR172 isoforms regulate different processes: miR172b is involved in flowering, while miR172c mainly regulates nodulation. Our data indicate that these miR172 isoforms exert their effects by silencing different target genes from the AP2 TF superfamily. Transcripts from two *AP2* genes (Phvul.005G138300 and Phvul.011G071100) are likely to function in roots and are cleaved by miR172c in nodules. Three other *AP2* genes (Phvul.003G241900, Phvul.002G16900, and Phvul.001G174400) are likely to function in young flowers, which showed a high level of these transcripts as well as of miR172, thus suggesting that, in flowers, the *AP2* target genes are silenced by miR172-induced translation inhibition, similar to Arabidopsis (Chen, 2004). Our work focused on the analysis of the miR172c/*AP2-1* (Phvul.005G138300) node in the common bean-rhizobia nitrogen-fixing symbiosis, and our proposed regulatory model is summarized in Figure 10.

In Arabidopsis, miR156 represses miR172 expression by targeting *SPL* TFs that directly bind to the *MIR172*

Table II. *Selected genes coexpressed with AP2-1 from the statistically overrepresented GO:0004672 category: protein kinase activity*

Expression level was determined by qRT-PCR from 21-dpi mature nodules (N) and roots (R) of EV and OEAP2m *R. etli*-inoculated composite plants and from 18 dpi mature or 35 dpi senescent nodules (N) from *R. etli*-inoculated wild-type plants. Values represent averages ± SD from three independent biological replicates and two technical replicates. TFBS for ERF and DREB (subfamilies of the AP2 TF family) were identified as statistically overrepresented in the promoter sequence of each gene as indicated.

Gene Identifier[a]	Annotation[a]	TFBS	Expression Level					
			21 dpi				Wild Type	
			EV		OEAP2m		18 dpi	35 dpi
			R	N	R	N	N	N
Phvul.002G326600	Aminocyclopropane carboxylate oxidase	DREB	0.76 ± 0.08	0.35 ± 0.13	1.2 ± 0.06	1.03 ± 0.07	0.11 ± 0.01	1.03 ± 0.07
Phvul.007G049500	Ser/Thr protein kinase	ERF	0.61 ± 0.09	0.25 ± 0.03	0.93 ± 0.08	0.97 ± 0.09	0.10 ± 0.02	0.53 ± 0.05
Phvul.007G049000	Ser/Thr protein kinase	DREB	0.10 ± 0.007	0.043 ± 0.01	0.02 ± 0.003	0.03 ± 0.004	0.07 ± 0.009	0.21± 0.03
Phvul.006G174500	Glycogen synthase kinase-3α	DREB	0.45 ± 0.06	0.24 ± 0.03	0.71 ± 0.07	0.57 ± 0.06	0.16 ± 0.02	0.37 ± 0.03
Phvul.011G169600	Ser/Thr protein kinase	DREB	0.35 ± 0.02	0.18 ± 0.02	0.52 ± 0.03	0.47 ± 0.03	0.33 ± 0.03	0.36 ± 0.02
Phvul.008G263900	Ser/Thr protein kinase	ERF and DREB	1.2 ± 0.06	0.15 ± 0.04	0.57 ± 0.02	0.50 ± 0.09	0.0023 ± 0.0003	0.0019 ± 0.0002

[a]From http://www.phytozome.net/commonbean.php.

promoter and positively regulate its expression (Wu et al., 2009). Transgenic soybean roots overexpressing miR156 showed a reduction in nodulation, decreased miR172 level, and decreased expression of two *SPL* genes proposed as miR156 targets, although evidence for the binding of SPL to *MIR172* promoters for transcription activation was not provided (Yan et al., 2013). Recently, Wang et al. (2015) reported that the overexpression of miR156 affects several aspects of plant architecture in *L. japonicus*, including underdeveloped roots and reduced nodulation, which correlate with the repression of several early symbiotic genes. However, the authors did not analyze a possible regulation of miR172 by miR156, which may be related to the miR156 effects in nodulation that they showed (Wang et al., 2015). In common bean, we observed opposite levels of mature miR156a as compared with miR172 and also a negative correlation between miR156a and its validated target *SPL6* gene (Phvul.009G165100). However, we could not identify SPL TFBS in any of the promoter regions of the six *MIR172* loci from the common bean genome, so it is difficult to propose SPL as a direct transcriptional regulator of miR172. However, the binding of SPL proteins to yet unknown sequence motifs present in *MIR172* promoters cannot be ruled out. Alternatively, miR156a may exert its negative regulation over common bean miR172 through other target genes not yet identified. For example, transcripts coding for tryptophan-aspartic acid repeats protein domain proteins, which may be involved in microtubule organization, protein-protein and protein-DNA interactions, or chromatin conformation, have been validated as miR156 targets in *M. truncatula* and *L. japonicus*, but their specific regulatory function has not been analyzed (Naya et al., 2010; Wang et al., 2015).

In this work, we showed that miR172c has a positive effect on root development independent of rhizobium infection. In addition, miR172c is relevant for the control of rhizobial infection. This miRNA increased after 6 h in *R. etli*-inoculated roots, when infection threads are formed, and this is related to the increase in root hair deformation observed in plants that overexpress miR172c. Preliminary data indicate that the roots overexpressing AP2m induce irregular infection threads (B. Nova-Franco, O. Valdés-López, and G. Hernández, unpublished data). Together, these data indicate a regulatory/signaling role of miR172c in the rhizobial infection of common bean (Fig. 10). Up-regulation of miR172c was concomitant with that of early nodulation genes, mainly expressed in the cortical cells, that are involved in infection thread initiation/progression (i.e. *FLOT2* and *ENOD40*) and act downstream of *NSP2*, *NIN*, and *NF-YA1* (Murray, 2011; Oldroyd, 2013), whose expression was highest after 3 h of rhizobial inoculation of common bean roots. Therefore, we propose that miR172c-mediated control of rhizobial infection is exerted at the level of cortical cell division downstream of NF perception, Ca^{2+} spiking, CCaMK, NSP2, and NIN (Fig. 10). In addition, our data on the repression of *RIC1* in roots overexpressing miR172c indicate the involvement of this miRNA in the AON at early stages of the common bean symbiosis. Soyano et al. (2014) reported that the AON *L. japonicus CLE* root signal genes *CLE-RS1* and *CLE-RS2*, which are orthologous to soybean *RIC1* and *RIC2*, are directly transcribed by NIN, the essential inducer for nodule primordium formation. This constitutes a complex regulatory circuit with a long-distance feedback loop involved in the homeostatic regulation of nodule organ production in *L. japonicus* (Soyano et al., 2014). In soybean, Wang et al. (2014a) recently reported that NARK negatively regulates miR172 transcription during nodule primordium formation to prevent excess nodulation. In this work, we showed that both *NIN* and *RIC1* are expressed at early stages of rhizobial infection in common bean roots and that OEAP2 roots with decreased nodulation showed increased *RIC1* levels. Taken together, these data would indicate a positive regulation of *NIN* and *AP2-1* to *RIC1*, thus leading to reduced nodulation through AON in common bean (Fig. 10). Sequence analysis of the *RIC1* promoter region led us to identify NIN- and DREB/ERF-enriched regions, something that supports the latter contention. However, further work is required to fully demonstrate which TFs activate *RIC1* expression in common bean.

The regulation of nodulation through AON signaling is also relevant for the inhibition of nodulation that occurs when nitrate is present in the rhizosphere. For local nitrate inhibition, the nitrate-induced CLE peptide in soybean (NIC) interacts with NARK in the root, leading to a nitrate-induced inhibition of nodulation in soybean (Reid et al., 2011a). Our data indicate that common bean miR172c is a signaling component of the nitrate-induced AON (Fig. 10). In the presence of nitrate, rhizobia-inoculated roots that overexpress miR172c developed more active nodules and showed very low expression of *NIC1* that correlates with the up-regulation of *NF-YA1*, *NSP2*, *CYCLOPS*, *ENOD40*, and *FLOT2* early symbiotic genes. The expression of *NIN* was similar in EV and OE172 roots inoculated in the absence or presence of nitrate, which is in agreement with data from *L. japonicus* reported by Soyano et al. (2015). The legume-rhizobia symbiosis with increased resistance to soil nitrate is relevant for improving plant growth and crop production. Our better understanding of the elements involved in the control of this phenomenon, such as miR172c in common bean, opens the possibility to exploit it for the future improvement of symbiosis.

The effect of miR172c in rhizobial infection and nodulation of common bean is likely to be directly exerted by its target gene, the AP2-1 transcriptional regulator. TFs from the AP2 superfamily may function as repressors or as activators of transcription (Licausi et al., 2013). Work recently published by Yan et al. (2013) and Wang et al. (2014) about the mechanism of action of AP2 in soybean nodulation points to the repressor role of AP2. In this work, we explored the possible role of *AP2-1* as a transcriptional activator and/or repressor of genes relevant for common bean rhizobium infection and nodulation. Recently, Soyano et al. (2015) reported that in *L. japonicus*, the NIN TF could repress or activate transcription in different scenarios of rhizobial infection in the presence or

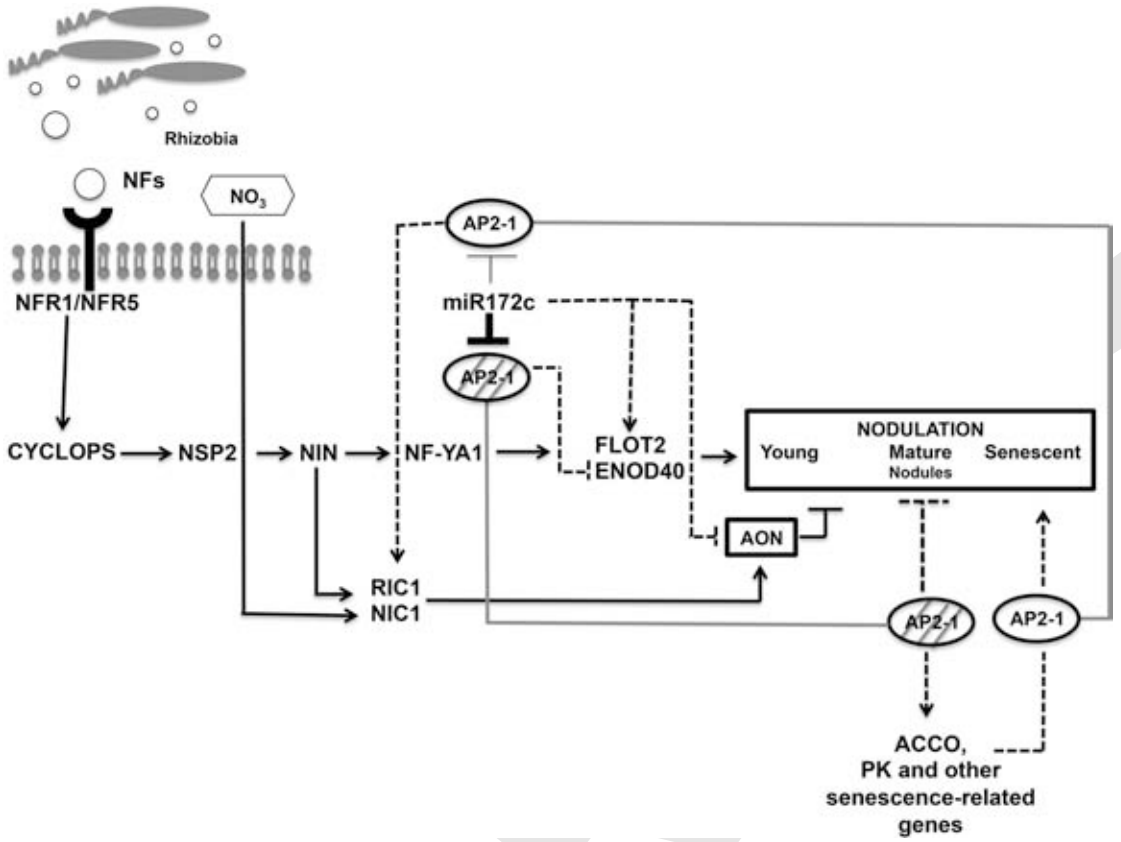


Figure 10. Model of miR172 node regulation in common bean-rhizobia symbiosis. Positive regulation is represented with arrows and negative regulation with lines. The root signaling cascade, triggered by rhizobial NF, is essential for rhizobia infection and nodule development in different legumes. Regulation of common bean rhizobial infection and nodulation by miR172c and AP2-1 are represented by dashed arrows or lines. A high level of miR172c (thick line) induces AP2-1 degradation (hatched circle), while active AP2-1 (circle) is present when the miR172c level is very low (thin line). miR172c positively regulates early nodulation gene expression and rhizobial infection, silencing AP2-1 that may repress *ENOD40* expression. Nodule number is positively regulated by miR172c; AON decreases through low RIC1/NIC1 expression, perhaps regulated by AP2-1. In mature nodules, abundant miR172c silences AP2-1, an activator of senescence-related genes that are further required during nodule senescence when AP2-1 levels are recovered. ACCO, Aminocyclopropane carboxylase oxidase.

absence of nitrate. They postulated that such dual regulatory functions might depend on specific coactivator or corepressor molecules that would interact with the same NIN TF in different scenarios.

We identified genes that significantly coexpress with *AP2-1* and are candidates for transcriptional activation by this TF (Fig. 10). Several of these genes were assigned to the protein kinase activity GO category. The best candidates are four protein kinases that are repressed in mature nodules and induced in roots and in senescent nodules of control plants, whereas they show high and/or constitutive expression in roots and nodules of AP2m overexpressing plants. This group includes the Phvul.007G049500 gene annotated (www.phytozome.net/commonbean.php) as a Ser/Thr protein kinase with a Cys-rich receptor-like protein kinase, Domain of Unknown Function26 (transmembrane), and Ser/Thr protein kinase domains. We found that this common bean kinase gene is similar (48%) to the Arabidopsis *CYSTEINE-RICH RECEPTOR-LIKE PROTEIN KINASE29* (*CRK29*) gene and to *M. truncatula SymCRK* (52% similarity) and has domains characteristic of the Cys-rich kinase family. Several members of this family are induced during *M. truncatula* nodule senescence (Van de Velde et al., 2006; Pérez Guerra et al., 2010). Specifically, SymCRK is involved in senescence and defense-like reactions during the *M. truncatula-Sinorhizobium meliloti* symbiosis (Berrabah et al., 2014). Another gene from this group encodes the aminocyclopropane carboxylase oxidase, the ethylene-forming enzyme. This and other genes encoding enzymes from the ethylene biosynthetic pathway are up-regulated during *M. truncatula* nodule senescence (Van de Velde et al., 2006). Ethylene plays a positive role in nodule senescence and also a significant inhibitory role in rhizobial infection and nodule formation (Van de Velde et al., 2006; Murray, 2011). Our interpretation of these results is that *AP2-1* transcriptional regulation is important in common bean roots but that this TF needs to be silenced for an adequate nodule function (SNF), something that is achieved

by posttranscriptional target cleavage mediated by miR172c. *AP2-1* silencing in effective mature nodules would maintain functionality by avoiding senescence through the down-regulation of nodule senescence genes activated by this TF (Fig. 10). Ineffective nodules where miR172c is not induced and AP2-1 remains elevated, as well as OEAP2 nodules, showed early senescence. Alternatively, other protein kinases, proposed as *AP2-1* targets, may participate in signaling pathways important for root development or nodule senescence. Nodule-specific protein kinases essential for signaling pathways during initial stages of nodulation are known (Oldroyd and Downie, 2008; Kouchi et al., 2010; Murray, 2011; Oldroyd, 2013).

Regarding AP2 transcriptional repression, Wang et al. (2014) recently reported that soybean NNC1, a miR172 target, represses *ENOD40* expression, which results in negative regulation of early stages of rhizobial symbiosis. In this work, we showed that the expression of common bean *ENOD40* is decreased in rhizobia-inoculated roots that overexpress AP2m. In addition, we identified ERF- and DREB-enriched regions in the *ENOD40* 5′ promoter region. So it is conceivable that, as in soybean, common bean *ENOD40* expression is repressed by AP2-1 (Fig. 10). However, it is important to consider that soybean *NNC1* is not the ortholog of common bean *AP2-1*. We identified *NNC1* (glyma12g07800) as the ortholog (84% similarity) of the Phvul.011G071100 *AP2* gene, identified as a miR172 target in a common bean degradome analysis (D. Formey, L.P. Íñiguez, P. Peláez, Y.F. Li, R. Sunkar, F. Sánchez, J.L. Reyes, and G. Hernández, unpublished data) but not further analyzed in this work; while common bean *AP2-1* was identified as the ortholog (93% similarity) of the soybean *AP2* gene glyma15g04930 that was predicted but not validated as the miR172 target (Wang et al., 2014). Therefore, different miR172 target genes from the AP2 family analyzed in soybean and in common bean may have different mechanisms for transcriptional regulation.

In addition, Yan et al. (2013) postulated that the regulation of soybean nodulation by miR172 is explained by the *AP2* repression of nonsymbiotic hemoglobin (*Hb*) gene expression that is essential for regulating the level of nodulation; however, the authors did not provide evidence demonstrating AP2 binding and direct transcriptional repression of *Hb* genes. To explore if this circuit is functioning in common bean, we first identified *Hb* genes encoded by the common bean genome: five symbiotic leg-hemoglobin (*Lb*) genes having greatly increased expression in effective nodules and four nonsymbiotic *Hb* genes (O'Rourke et al., 2014; Supplemental Fig. S7). From the latter, Phvul011G048600 and Phvul.011G048700 were identified as orthologs of the nonsymbiotic *Hb-1* and *Hb-2* genes in soybean, respectively. In common bean, these *Hb* genes showed similar low expression in roots and nodules of wild-type plants and also in composite plants that overexpress *AP2-1* or that have very low AP2-1 resulting from miR172 overexpression (Supplemental Fig. S7). Therefore, our data differ from those of Yan et al. (2013) and lead us to conclude that *AP2-1* repression of *Hb-1* genes is not relevant for common bean nodulation. Our exploration of other common bean symbiotic genes repressed by *AP2-1* included the identification of TFBS statistically overrepresented in the promoter regions of 402 common bean genes reported by O'Rourke et al. (2014) as nodule-enhanced genes. The expression pattern of these genes is similar to that of miR172c and opposite to that of *AP2-1*, which shows low expression in mature nodules and high expression in roots, suggesting these as candidates for transcriptional repression by AP2-1. However, AP2 (ERF and DREB) TFBS were not overrepresented in these genes. The latter is different from our data from *AP2-1* coexpressed genes proposed as being activated by this TF; these genes did show overrepresentation of ERF/DREB TFBS in their promoter regions. Further work is required to demonstrate the direct transcription repression, if any, of the miR172c target gene *AP2-1* in common bean.

Legume crops with increased nodulation/decreased nitrate inhibition of nodulation would be relevant for sustainable agriculture. This work sets the basis for further exploration, through genetic/genomic approaches, for common bean cultivars with improved traits resulting from increased miR172 in roots and nodules.

MATERIALS AND METHODS

Identification and Analysis of miR172 Precursor Genes, Isoforms, and Target Genes

The common bean (*Phaseolus vulgaris*) genome sequence recently published (Schmutz et al., 2014; http://www.phytozome.net/commonbean.php, v1.0) was analyzed, and six miR172 isoforms (a–f) were identified. Of these, four isoforms were described previously through RNA-seq analysis of common bean small RNAs by Peláez et al. (2012). The secondary RNA structure of each miR172 isoform was predicted using mfold software (Zuker, 2003) available at http://mfold.ma.albany.edu, and only the lowest energy structure generated for each sequence was chosen (Supplemental Fig. S1).

Since the only targets identified for miR172 from different plant species belong to the *AP2*-type TF family, we focused our analysis on identifying common bean miR172 targets within this gene family. We performed target prediction analysis for all the common bean *AP2* gene transcripts identified in the *Pv* GEA (O'Rourke, et al., 2014) using the Web server psRNATarget (http://plantgrn.noble.org/psRNATarget/; Dai and Zhao, 2011). Stringent criteria were used to predict targets; that is, an alignment spanning at least 18 bp with maximum penalty score of 3. Score calculation considered 0.5 points for each G:U wobble, one point for each non-G:U mismatch, and two points for each bulged nucleotide in either RNA strand (Jones-Rhoades and Bartel., 2004). In addition, we constructed a phylogenetic cladogram from the amino acid sequences of common bean *AP2* genes; these were aligned using ClustalX version 2.1 (Larkin et al., 2007). The sequence-aligned file was used to construct the bootstrapped neighbor-joining tree using the NJ clustering algorithm and Phylip output format (.phb). The reliability of the phylogenetic analysis was estimated from 1,000 bootstrap resamplings, and the tree was viewed using the program MEGA version 5.2.1 (Tamura et al., 2011).

Plant Material and Growth Conditions

Common bean seeds from the Mesoamerican cv Negro Jamapa 81 were surface sterilized and germinated for 2 d at 26°C to 28°C in darkness. Plants were grown in a hydroponic system under controlled environmental conditions as described previously (Valdés-López et al., 2010). The hydroponic trays contained 8 L of Franco and Munns (1982) nutrient solution. The volume and pH (6.5) of the trays were controlled throughout the experiment. For SNF conditions, plantlets adapted by growth for 7 d in the hydroponic system with

nitrogen-free nutrient solution were inoculated with 200 mL of a saturated liquid culture of the *Rhizobium etli* CE3 wild-type strain or the *R. etli fix*$^-$ mutant strain CFNX247 (Δ*nifA*::ΩSp/Sm; Girard et al., 1996). Plants were harvested at different times (dpi) for analysis; tissues for RNA isolation were collected directly into liquid nitrogen and stored at −80°C.

To analyze the initial events of rhizobial infection, 2-d-old seedlings were placed in plastic square bioassay dishes (24 × 24 cm; Corning) with solidified nitrogen-free Fähreus medium (Vincent, 1970). Plates containing common bean seedlings were incubated in a growth chamber at 25°C with a 16-h photoperiod. After 2 d, seedlings were inoculated by applying 1 mL of *R. etli* CE3 saturated liquid culture directly to the root and were further incubated at various times (3–48 h). After specific incubation times, the root responsive zones were detached, frozen in liquid nitrogen, and stored at −80°C until used.

Plasmid Construction, Plant Transformation, and Production of Composite Plants

To generate a plasmid to overexpress the pre-miR172 in common bean transgenic roots, a 217-bp PCR product was obtained using common bean nodule complementary DNA (cDNA) as template and the specific primers Fw-pre172 (5′-CACCCCAGTCACTGTTTGCCGGTGGAG-3′) and R-pre172 (5′-AAAAACCTCCTTTGCTCTGAGCGT-3′), based on the Phvul.001G233200 sequence. The PCR product was cloned by T-A annealing into pCR 2.1-TOPO (Invitrogen) and sequenced. To construct the OE172 plasmid, the pre-miR172 region was excised using the *Xho*I and *Bam*HI sites of the vector and cloned into the pTDTO plasmid that carries the reporter tdTomato (red fluorescent protein) gene (Aparicio-Fabre et al., 2013; Supplemental Fig. S3).

The complete cDNA clone of common bean *AP2-1* was obtained by PCR amplification using cDNA from roots and the specific primers Fw-AP2 (5′-CAGCTACCTTTCCGCCAAATGC-3′) and Rv-AP2 (5′-TAGGCTGGGATGGTGTCTGCAG-3′), based on the Phvul.005G138300 sequence. The 1,127-bp product was cloned by T-A annealing into pCR 2.1 TOPO (Invitrogen) and analyzed by sequencing. Mutations of the putative miR172 cleavage site of *AP2-1* were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The *Pst*I site present in the wild-type miR172 cleavage site (5′-CTGCAGCATCATCAGGATTCT-3′) was eliminated by changing the A to a C at the 3′ position of the recognition site for this enzyme. Additionally, the nucleotides at positions 9, 10, and 11 of the miR172 cleavage site were modified by introducing a *Bgl*II site. The primers used were forward (5′-TTCTCTACTGCCGCCAGATCTGGATTCTCAATT-3′) and reverse (5′-AATTGAGAATCCAGATCTGGCGGCAGTAGAGAA-3′). The changes were checked by sequencing, and the modified AP2 was cloned into plasmid pTDTO to obtain plasmid OEAP2m. The nucleotide changes in AP2m introduced an amino acid substitution (Arg for Ser), but this does not seem to affect AP2-1 function (Supplemental Fig. S3).

Common bean composite plants with transformed root system and untransformed aerial system were generated as described (Estrada-Navarrete et al., 2007; Aparicio-Fabre et al., 2013). For plant transformation, *Agrobacterium rhizogenes* K599 strains bearing the EV, OE172, or OEAP2m plasmids were used. Selected composite plants were grown under controlled environmental conditions in pots with vermiculite and watered daily with B&D nutrient solution (Broughton and Dilworth, 1971), either nitrogen free for the symbiotic condition or with 10 mM potassium nitrate for the full-nutrient condition. SNF plants were adapted by growing in pots for 7 d and then inoculated with *R. etli* CE3. For experiments designed to analyze the effect of nitrate on symbiosis, B&D nutrient solution supplemented with 1 or 3 mM KNO_3 was used to water the inoculated plants daily from 1 dpi. Composite plants were analyzed phenotypically at different dpi; transgenic roots and nodules were collected in liquid nitrogen and stored at −80°C.

To analyze the initial events of rhizobial infection in transgenic roots, plastic square bioassay dishes were used to grow selected composite plants under the same conditions described above. Plates containing composite plants were sealed with Parafilm, and the root zone was covered with aluminum foil. After 2 d, composite plants were inoculated by applying 1 mL of *R. etli* CE3 saturated liquid culture directly to the root. At 48 hpi, the root responsive zones were detached and stored at −80°C until used or collected into phosphate-buffered saline (PBS) buffer for microscopic analyses.

Phenotypic Analysis

Nitrogenase activity was determined in detached nodulated roots by the acetylene reduction assay essentially as described by Hardy et al. (1968). Specific activity is expressed as nmol ethylene h^{-1} g^{-1} nodule dry weight.

The root fresh weight and the number of secondary roots per plant were determined in composite plants grown under full-nutrient (10 d) or symbiotic (21 dpi) conditions.

Microscopic analysis was performed on transgenic nodules at different developmental stages from EV, OE172, and OEAP2m composite plants. The protocol described by Haynes et al. (2004) was used for tissue staining with the nucleic acid-binding dye SYTO13. Nodule sections were stained with SYTO13 (1 μL mL^{-1}) in 80 mM PIPES, pH 7, for 5 min, then mounted in 1% (v/v) PBS/50% (v/v) glycerol and analyzed. Images were obtained using the Zeiss LSM 510 laser scanning microscope attached to an Axiovert 200 M. SYTO13 excitation was obtained at 488 nm using an argon laser and an HFT UV 488/543/633-nm dual dichroic excitation mirror with an LP 560 emission filter for detection. Sequentially, red fluorescence from the reporter gene was observed by exciting at 543 nm with a helium/neon laser, with the same dual dichroic excitation mirror and a BP 500-530 IR emission filter. Images were processed using the LSM 510 version 4.2 5P1 software (Carl Zeiss Micro-Imaging). For the determination of nodule perimeter and SYTO13 intensity per infected area, 10 images from individual nodule replicates from each condition were analyzed using the ImageJ program.

Statistical analyses of symbiotic parameters (root biomass/architecture, nodulation, and nitrogenase activity) were performed using one-way ANOVA and multiple paired Student's *t* tests ($P < 0.001$).

For analyses of root hair deformation and infection thread induction by rhizobial infection, the root responsive zones from inoculated composite plants grown in plastic square bioassay dishes, as described above, were collected at 48 hpi into PBS buffer. Responsive zone root samples were stained with 0.01% (w/v) Methylene Blue for 1 h and washed three times with double-distilled water; infection events were observed in an optical microscope.

RNA Isolation and Analysis

Total RNA was isolated from 100 mg of frozen nodules, 250 mg of frozen roots, or 200 mg of other frozen tissues from wild-type or composite plants grown under similar conditions, using Trizol reagent (Life Technologies) following the manufacturer's instructions. These samples were preserved at −80°C until tested. Genomic DNA removal, cDNA synthesis, and quality verification for qRT-PCR were performed as reported (Hernández et al., 2007).

RNA preparations were used to detect mature miR172 in different plant tissues by low-molecular-weight RNA-gel hybridization using 15 μg of total RNA, as reported (Naya et al., 2014). Synthetic DNA oligonucleotides with antisense sequence corresponding to miR172 (5′-ATGCAGCATCATCAAGATTCT-3′) and to U6 snRNA (5′-CCAATTTTATCGGATGTCCCCG-3′) were used as probes after radioactive labeling. Hybridization of U6 snRNA was used as a loading control. The signal intensities of miR172 and U6 hybridization bands were determined using ImageQuant 5.2 software (Molecular Dynamics). Normalized miR172 expression levels were calculated related to U6 snRNA.

For the quantification of transcript levels of mature miRNAs, cDNA was synthesized from 1 μg of total RNA using the NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen) or the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) for transcripts of selected genes. Resulting cDNAs were then diluted and used to perform qRT-PCR assays using SYBR Green PCR Master Mix (Applied Biosystems), following the manufacturer's instructions. The sequences of oligonucleotide primers used for qRT-PCR amplification of each gene are provided in Supplemental Table S1. Reactions were analyzed in a real-time thermocycler (Eco Illumina Real-Time PCR System; Illumina) with settings of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 57°C for 60 s. Relative expression for each sample was calculated with the comparative C_t method. The C_t value obtained after each reaction was normalized with the C_t value of miR159 for miRNA levels or with the C_t value of *UBC* (Phvul.006G110100) for expression levels of miRNAs and transcripts, respectively.

Statistical analyses of gene expression (miR172c, *AP2-1*, and early symbiotic genes) from wild-type and composite SNF plants were performed using one-way ANOVA and multiple paired Student's *t* tests ($P < 0.001$).

Identification of Root-Enhanced Genes Coexpressed with *AP2-1*

To identify common bean genes with an expression pattern similar to that of the *AP2-1* target gene, the Euclidian distance between Z scores for each gene was determined in RNA-seq samples from the *Pv* GEA (O'Rourke et al., 2014). The tissue samples analyzed were as follows: young roots, prefixing effective

(5 dpi) nodules, effective (21 dpi) nodules, ineffective (21 dpi) nodules, roots from nonsymbiotic plants grown in full-nutrient solution, nodule-detached roots (5 dpi), effective nodule-detached roots (21 dpi), and ineffective nodule-detached roots (21 dpi). A threshold Euclidian distance of 0.9 was established as significant. A total of 114 genes within the threshold were identified as genes coexpressed with *AP2-1* (Supplemental Table S5).

Gene Sequence Analysis for the Identification of TFBS

The CLOVER program (Frith et al., 2004) was used to identify TFBS in 5′ promoter regions of *AP2-1* coexpressed genes (Supplemental Table S5) and of genes highly expressed in mature effective nodules described previously by O'Rourke et al. (2014). For this analysis, a 2,000-bp sequence from the region immediately upstream of the transcription start site of each gene was retrieved from the common bean genome sequence (Schmutz et al., 2014; http://www.phytozome.net/commonbean.php, v1.0).

Promoter regions (2,000-bp sequence) of selected early nodulation genes were tested for DREB/ERF or NIN binding sites using http://plants.rsat.eu/. A Markov order of 2 was used for predicting cis-regulatory element-enriched regions with default parameters. The cis-regulatory element-enriched regions were also searched in the promoter region of each locus encoding an miR172 isoform; 1,000 bp upstream of the transcription start site of isoforms a and c and 1,500 bp upstream of the precursors of isoforms b, d, e, and f were analyzed (Supplemental Table S2).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. miR172 isoforms encoded in the common bean genome and the most stable secondary structures predicted for their precursors.

Supplemental Figure S2. Symbiotic phenotypes of common bean plants inoculated with the *R. etli nifA*$^-$ mutant strain as compared with the CE3 wild-type strain.

Supplemental Figure S3. Schematic representation of plasmids used for miR172c or *AP2-1m* overexpression.

Supplemental Figure S4. Overexpression of miR172c and *AP2m* in transgenic roots and nodules of composite bean plants.

Supplemental Figure S5. miR172 and *AP2-1* control rhizobial infection in common bean roots.

Supplemental Figure S6. Expression pattern of *AP2-1* coexpressed genes.

Supplemental Figure S7. Expression analysis of common bean symbiotic (*Lb*) and nonsymbiotic (*Hb*) hemoglobin genes.

Supplemental Table S1. Primer sequences for qRT-PCR.

Supplemental Table S2. TFBS identified in the 5′ promoter region of each *MIR172* gene.

Supplemental Table S3. Nitrogenase activity and expression analysis of marker genes for nodule development.

Supplemental Table S4. Expression analysis of marker genes for nodule development in transgenic nodules of OE172, EV, or OEAP2m plants.

Supplemental Table S5. *AP2-1* coexpressed genes: assigned GO categories and identified TFBS.

ACKNOWLEDGMENTS

We thank Dr. Jesús Arellano (Universidad Autónoma del Estado de Morelos [UAEM]) and María del Socorro Sánchez Correa (Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México) for technical assistance in plant transformation, Dr. Ramón Suárez (UAEM) for cooperation in qRT-PCR determinations, Darla Boydston (Noble Foundation) for helping in the graphic design of the figures, and Dr. Michael Dunn (Centro de Ciencias Genómicas-Universidad Nacional Autónoma de México) for critically reviewing the article.

Received December 12, 2014; accepted March 2, 2015; published March 4, 2015.

LITERATURE CITED

Aparicio-Fabre R, Guillén G, Loredo M, Arellano J, Valdés-López O, Ramírez M, Iñiguez LP, Panzeri D, Castiglioni B, Cremonesi P, et al (2013) Common bean (*Phaseolus vulgaris* L.) PvTIFY orchestrates global changes in transcript profile response to jasmonate and phosphorus deficiency. BMC Plant Biol **13:** 26

Arenas-Huertero C, Pérez B, Rabanal F, Blanco-Melo D, De la Rosa C, Estrada-Navarrete G, Sánchez F, Covarrubias AA, Reyes JL (2009) Conserved and novel miRNAs in the legume *Phaseolus vulgaris* in response to stress. Plant Mol Biol **70:** 385–401

Ariel F, Brault-Hernandez M, Laffont C, Huault E, Brault M, Plet J, Moison M, Blanchet S, Ichanté JL, Chabaud M, et al (2012) Two direct targets of cytokinin signaling regulate symbiotic nodulation in *Medicago truncatula*. Plant Cell **24:** 3838–3852

Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a microRNA and its *APETALA2*-like target genes. Plant Cell **15:** 2730–2741

Bazin J, Bustos-Sanmamed P, Hartmann C, Lelandais-Brière C, Crespi M (2012) Complexity of miRNA-dependent regulation in root symbiosis. Philos Trans R Soc Lond B Biol Sci **367:** 1570–1579

Berrabah F, Bourcy M, Eschstruth A, Cayrel A, Guefrachi I, Mergaert P, Wen J, Jean V, Mysore KS, Gourion B, et al (2014) A nonRD receptor-like kinase prevents nodule early senescence and defense-like reactions during symbiosis. New Phytol **203:** 1305–1314

Boualem A, Laporte P, Jovanovic M, Laffont C, Plet J, Combier JP, Niebel A, Crespi M, Frugier F (2008) MicroRNA166 controls root and nodule development in *Medicago truncatula*. Plant J **54:** 876–887

Broughton WJ, Dilworth MJ (1971) Control of leghaemoglobin synthesis in snake beans. Biochem J **125:** 1075–1080

Broughton WJ, Hernández G, Blair M, Beebe S, Gepts P, Vanderleyden J (2003) Beans (*Phaseolus* spp.): model food legumes. Plant Soil **252:** 55–128

Bustos-Sanmamed P, Mao G, Deng Y, Elouet M, Khan GA, Bazin J, Lelandais-Brière C (2013) Overexpression of miR160 affects root growth and nitrogen-fixing nodule number in *Medicago truncatula*. Funct Plant Biol **40:** 1208–1220

Chen X (2004) A microRNA as a translational repressor of APETALA2 in *Arabidopsis* flower development. Science **303:** 2022–2025

Combier JP, Frugier F, de Billy F, Boualem A, El-Yahyaoui F, Moreau S, Vernié T, Ott T, Gamas P, Crespi M, et al (2006) MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. Genes Dev **20:** 3084–3088

Contreras-Cubas C, Rabanal FA, Arenas-Huertero C, Ortiz MA, Covarrubias AA, Reyes JL (2012) The Phaseolus vulgaris miR159a precursor encodes a second differentially expressed microRNA. Plant Mol Biol **80:** 103–115

Crespi M, Frugier F (2008) De novo organ formation from differentiated cells: root nodule organogenesis. Sci Signal **1:** re11

Dai X, Zhao PX (2011) psRNATarget: a plant small RNA target analysis server. Nucleic Acids Res **39:** W155–W159

De Luis A, Markmann K, Cognat V, Holt DB, Charpentier M, Parniske M, Stougaard J, Voinnet O (2012) Two microRNAs linked to nodule infection and nitrogen-fixing ability in the legume *Lotus japonicus*. Plant Physiol **160:** 2137–2154

D'haeseleer K, Den Herder G, Laffont C, Plet J, Mortier V, Lelandais-Brière C, De Bodt S, De Keyser A, Crespi M, Holsters M, et al (2011) Transcriptional and post-transcriptional regulation of a NAC1 transcription factor *in Medicago truncatula* roots. New Phytol **191:** 647–661

Dong Z, Shi L, Wang Y, Chen L, Cai Z, Wang Y, Jin J, Li X (2013) Identification and dynamic regulation of microRNAs involved in salt stress responses in functional soybean nodules by high-throughput sequencing. Int J Mol Sci **14:** 2717–2738

Estrada-Navarrete G, Alvarado-Affantranger X, Olivares JE, Guillén G, Díaz-Camino C, Campos F, Quinto C, Gresshoff PM, Sanchez F (2007) Fast, efficient and reproducible genetic transformation of *Phaseolus* spp. by *Agrobacterium rhizogenes*. Nat Protoc **2:** 1819–1824

Ferguson BJ, Indrasumunar A, Hayashi S, Lin MH, Lin YH, Reid DE, Gresshoff PM (2010) Molecular analysis of legume nodule development and autoregulation. J Integr Plant Biol **52:** 61–76

Formey D, Sallet E, Lelandais-Brière C, Ben C, Bustos-Sanmamed P, Niebel A, Frugier F, Combier JP, Debellé F, Hartmann C, et al (2014) The small RNA diversity from *Medicago truncatula* roots under biotic

interactions evidences the environmental plasticity of the miRNAome. Genome Biol **15:** 457

Franco AA, Munns DN (1982) Nodulation and growth of *Phaseolus vulgaris* in solution culture. Plant Soil **66:** 149–160

Frith MC, Fu Y, Yu L, Chen JF, Hansen U, Weng Z (2004) Detection of functional DNA motifs via statistical over-representation. Nucleic Acids Res **32:** 1372–1381

Girard L, Valderrama B, Palacios R, Romero D, Dávila G (1996) Transcriptional activity of the symbiotic plasmid of *Rhizobium etli* is affected by different environmental conditions. Microbiology **142:** 2847–2856

Hardy RW, Holsten RD, Jackson EK, Burns RC (1968) The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol **43:** 1185–1207

Haynes JG, Czymmek KJ, Carlson CA, Veereshlingam H, Dickstein R, Sherrier DJ (2004) Rapid analysis of legume root nodule development using confocal microscopy. New Phytol **163:** 661–668

Hernández G, Ramírez M, Valdés-López O, Tesfaye M, Graham MA, Czechowski T, Schlereth A, Wandrey M, Erban A, Cheung F, et al (2007) Phosphorus stress in common bean: root transcript and metabolic responses. Plant Physiol **144:** 752–767

Huijser P, Schmid M (2011) The control of developmental phase transitions in plants. Development **138:** 4117–4129

Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell **14:** 787–799

Jung JH, Seo YH, Seo PJ, Reyes JL, Yun J, Chua NH, Park CM (2007) The *GIGANTEA*-regulated microRNA172 mediates photoperiodic flowering independent of *CONSTANS* in *Arabidopsis*. Plant Cell **19:** 2736–2748

Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA, Carrington JC (2003) P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. Dev Cell **4:** 205–217

Kouchi H, Imaizumi-Anraku H, Hayashi M, Hakoyama T, Nakagawa T, Umehara Y, Suganuma N, Kawaguchi M (2010) How many peas in a pod? Legume genes responsible for mutualistic symbioses underground. Plant Cell Physiol **51:** 1381–1397

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al (2007) Clustal W and Clustal X version 2.0. Bioinformatics **23:** 2947–2948

Lauressergues D, Delaux PM, Formey D, Lelandais-Brière C, Fort S, Cottaz S, Bécard G, Niebel A, Roux C, Combier JP (2012) The microRNA miR171h modulates arbuscular mycorrhizal colonization of *Medicago truncatula* by targeting NSP2. Plant J **72:** 512–522

Lelandais-Brière C, Naya L, Sallet E, Calenge F, Frugier F, Hartmann C, Gouzy J, Crespi M (2009) Genome-wide *Medicago truncatula* small RNA analysis revealed novel microRNAs and isoforms differentially regulated in roots and nodules. Plant Cell **21:** 2780–2796

Li H, Deng Y, Wu T, Subramanian S, Yu O (2010) Misexpression of miR482, miR1512, and miR1515 increases soybean nodulation. Plant Physiol **153:** 1759–1770

Licausi F, Ohme-Takagi M, Perata P (2013) APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. New Phytol **199:** 639–649

Magori S, Kawaguchi M (2009) Long-distance control of nodulation: molecules and models. Mol Cells **27:** 129–134

Martin A, Adam H, Díaz-Mendoza M, Zurczak M, González-Schain ND, Suárez-López P (2009) Graft-transmissible induction of potato tuberization by the microRNA miR172. Development **136:** 2873–2881

Murray JD (2011) Invasion by invitation: rhizobial infection in legumes. Mol Plant Microbe Interact **24:** 631–639

Naya L, Khan GA, Sorin C, Hartmann C, Crespi M, Lelandais-Brière C (2010) Cleavage of a non-conserved target by a specific miR156 isoform in root apexes of *Medicago truncatula*. Plant Signal Behav **5:** 328–331

Naya L, Paul S, Valdés-López O, Mendoza-Soto AB, Nova-Franco B, Sosa-Valencia G, Reyes JL, Hernández G (2014) Regulation of copper homeostasis and biotic interactions by microRNA 398b in common bean. PLoS ONE **9:** e84416

Okamoto S, Ohnishi E, Sato S, Takahashi H, Nakazono M, Tabata S, Kawaguchi M (2009) Nod factor/nitrate-induced *CLE* genes that drive HAR1-mediated systemic regulation of nodulation. Plant Cell Physiol **50:** 67–77

Oldroyd GED (2013) Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbiol **11:** 252–263

Oldroyd GED, Downie JA (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol **59:** 519–546

O'Rourke JA, Iniguez LP, Fu F, Bucciarelli B, Miller SS, Jackson SA, McClean PE, Li J, Dai X, Zhao PX, et al (2014) An RNA-Seq based gene expression atlas of the common bean. BMC Genomics **15:** 866

Peláez P, Trejo MS, Iñiguez LP, Estrada-Navarrete G, Covarrubias AA, Reyes JL, Sánchez F (2012) Identification and characterization of microRNAs in *Phaseolus vulgaris* by high-throughput sequencing. BMC Genomics **13:** 83

Pérez Guerra JC, Coussens G, De Keyser A, De Rycke R, De Bodt S, Van De Velde W, Goormachtig S, Holsters M (2010) Comparison of developmental and stress-induced nodule senescence in *Medicago truncatula*. Plant Physiol **152:** 1574–1584

Ramírez M, Graham MA, Blanco-López L, Silvente S, Medrano-Soto A, Blair MW, Hernández G, Vance CP, Lara M (2005) Sequencing and analysis of common bean ESTs: building a foundation for functional genomics. Plant Physiol **137:** 1211–1227

Reid DE, Ferguson BJ, Gresshoff PM (2011a) Inoculation- and nitrate-induced CLE peptides of soybean control NARK-dependent nodule formation. Mol Plant Microbe Interact **24:** 606–618

Reid DE, Ferguson BJ, Hayashi S, Lin YH, Gresshoff PM (2011b) Molecular mechanisms controlling legume autoregulation of nodulation. Ann Bot (Lond) **108:** 789–795

Rogers K, Chen X (2013) Biogenesis, turnover, and mode of action of plant microRNAs. Plant Cell **25:** 2383–2399

Rubio-Somoza I, Weigel D (2011) MicroRNA networks and developmental plasticity in plants. Trends Plant Sci **16:** 258–264

Schmid M, Uhlenhaut NH, Godard F, Demar M, Bressan R, Weigel D, Lohmann JU (2003) Dissection of floral induction pathways using global expression analysis. Development **130:** 6001–6012

Schmutz J, McClean PE, Mamidi S, Wu GA, Cannon SB, Grimwood J, Jenkins J, Shu S, Song Q, Chavarro C, et al (2014) A reference genome for common bean and genome-wide analysis of dual domestication. Nat Genet **46:** 707–713

Simon SA, Meyers BC, Sherrier DJ (2009) MicroRNAs in the rhizobia legume symbiosis. Plant Physiol **151:** 1002–1008

Song QX, Liu YF, Hu XY, Zhang WK, Ma B, Chen SY, Zhang JS (2011) Identification of miRNAs and their target genes in developing soybean seeds by deep sequencing. BMC Plant Biol **11:** 5

Soyano T, Hirakawa H, Sato S, Hayashi M, Kawaguchi M (2014) Nodule inception creates a long-distance negative feedback loop involved in homeostatic regulation of nodule organ production. Proc Natl Acad Sci USA **111:** 14607–14612

Soyano T, Shimoda Y, Hayashi M (2015) NODULE INCEPTION antagonistically regulates gene expression with nitrate in *Lotus japonicus*. Plant Cell Physiol **56:** 368–376

Streeter J, Wong PP (1988) Inhibition of legume nodule formation and N_2 fixation by nitrate. Crit Rev Plant Sci **7:** 1–23

Subramanian S, Fu Y, Sunkar R, Barbazuk WB, Zhu JK, Yu O (2008) Novel and nodulation-regulated microRNAs in soybean roots. BMC Genomics **9:** 160

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol **28:** 2731–2739

Turner M, Nizampatnam NR, Baron M, Coppin S, Damodaran S, Adhikari S, Arunachalam SP, Yu O, Subramanian S (2013) Ectopic expression of miR160 results in auxin hypersensitivity, cytokinin hyposensitivity, and inhibition of symbiotic nodule development in soybean. Plant Physiol **162:** 2042–2055

Turner M, Yu O, Subramanian S (2012) Genome organization and characteristics of soybean microRNAs. BMC Genomics **13:** 169

Valdés-López O, Arenas-Huertero C, Ramírez M, Girard L, Sánchez F, Vance CP, Luis Reyes J, Hernández G (2008) Essential role of MYB transcription factor: PvPHR1 and microRNA: PvmiR399 in phosphorus-deficiency signalling in common bean roots. Plant Cell Environ **31:** 1834–1843

Valdés-López O, Yang SS, Aparicio-Fabre R, Graham PH, Reyes JL, Vance CP, Hernández G (2010) MicroRNA expression profile in common bean (Phaseolus vulgaris) under nutrient deficiency stresses and manganese toxicity. New Phytol **187:** 805–818

Van de Velde W, Guerra JCP, De Keyser A, De Rycke R, Rombauts S, Maunoury N, Mergaert P, Kondorosi E, Holsters M, Goormachtig S (2006) Aging in legume symbiosis: a molecular view on nodule senescence in *Medicago truncatula*. Plant Physiol **141:** 711–720

Vincent JMA (1970) Manual for the Practical Study of Root Nodule Bacteria. IBP Handbook No. 15, Blackwell Scientific Publications, Edinburgh

Wang Y, Li P, Cao X, Wang X, Zhang A, Li X (2009) Identification and expression analysis of miRNAs from nitrogen-fixing soybean nodules. Biochem Biophys Res Commun **378:** 799–803

Wang Y, Wang L, Zou Y, Chen L, Cai Z, Zhang S, Zhao F, Tian Y, Jiang Q, Ferguson BJ, et al (2014) Soybean miR172c targets the repressive AP2 transcription factor NNC1 to activate *ENOD40* expression and regulate nodule initiation. Plant Cell **26:** 4782–4801

Wang Y, Wang Z, Amyot L, Tian L, Xu Z, Gruber MY, Hannoufa A (2015) Ectopic expression of miR156 represses nodulation and causes morphological and developmental changes in *Lotus japonicus*. Mol Genet Genomics **290:** 471–478

Wu G, Park MY, Conway SR, Wang JW, Weigel D, Poethig RS (2009) The sequential action of miR156 and miR172 regulates developmental timing in *Arabidopsis*. Cell **138:** 750–759

Yamashino T, Yamawaki S, Hagui E, Ueoka-Nakanishi H, Nakamichi N, Ito S, Mizuno T (2013) Clock-controlled and FLOWERING LOCUS T (FT)-dependent photoperiodic pathway in *Lotus japonicus*. I. verification of the flowering-associated function of an FT homolog. Biosci Biotechnol Biochem **77:** 747–753

Yan Z, Hossain MS, Wang J, Valdés-López O, Liang Y, Libault M, Qiu L, Stacey G (2013) miR172 regulates soybean nodulation. Mol Plant Microbe Interact **26:** 1371–1377

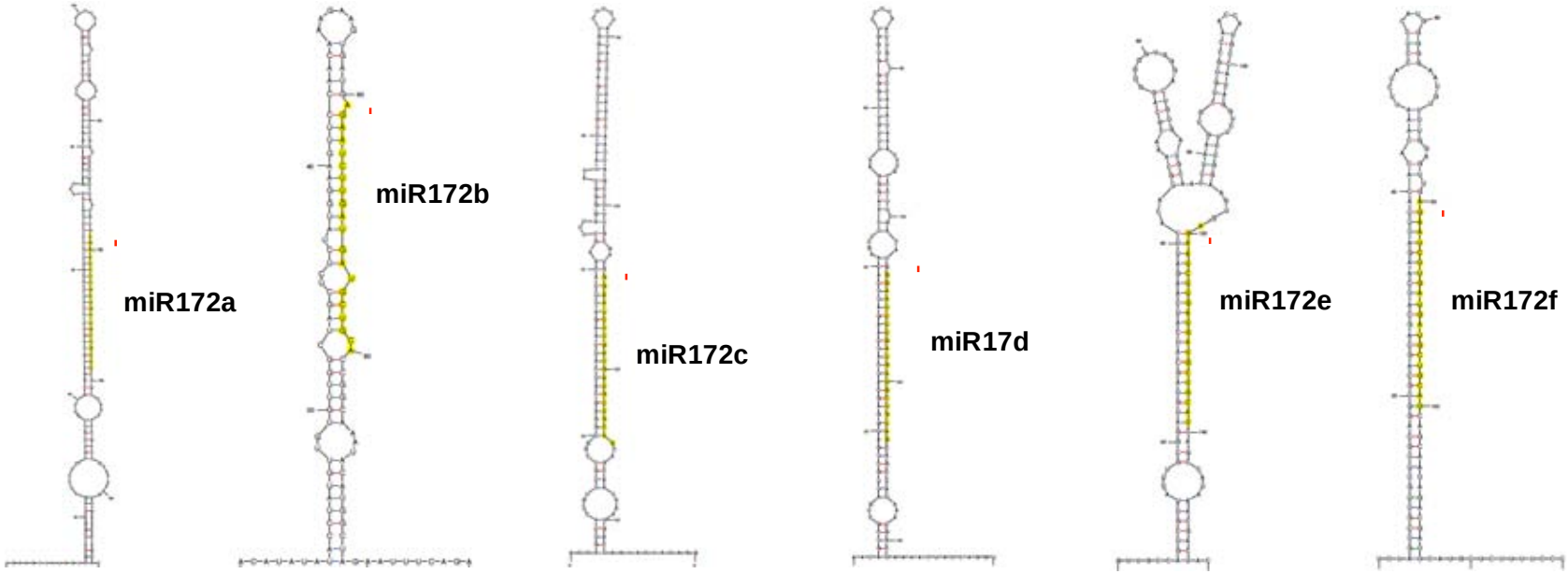
Yoshikawa T, Ozawa S, Sentoku N, Itoh J, Nagato Y, Yokoi S (2013) Change of shoot architecture during juvenile-to-adult phase transition in soybean. Planta **238:** 229–237

Zhang B, Pan X, Cobb GP, Anderson TA (2006) Plant microRNA: a small regulatory molecule with big impact. Dev Biol **289:** 3–16

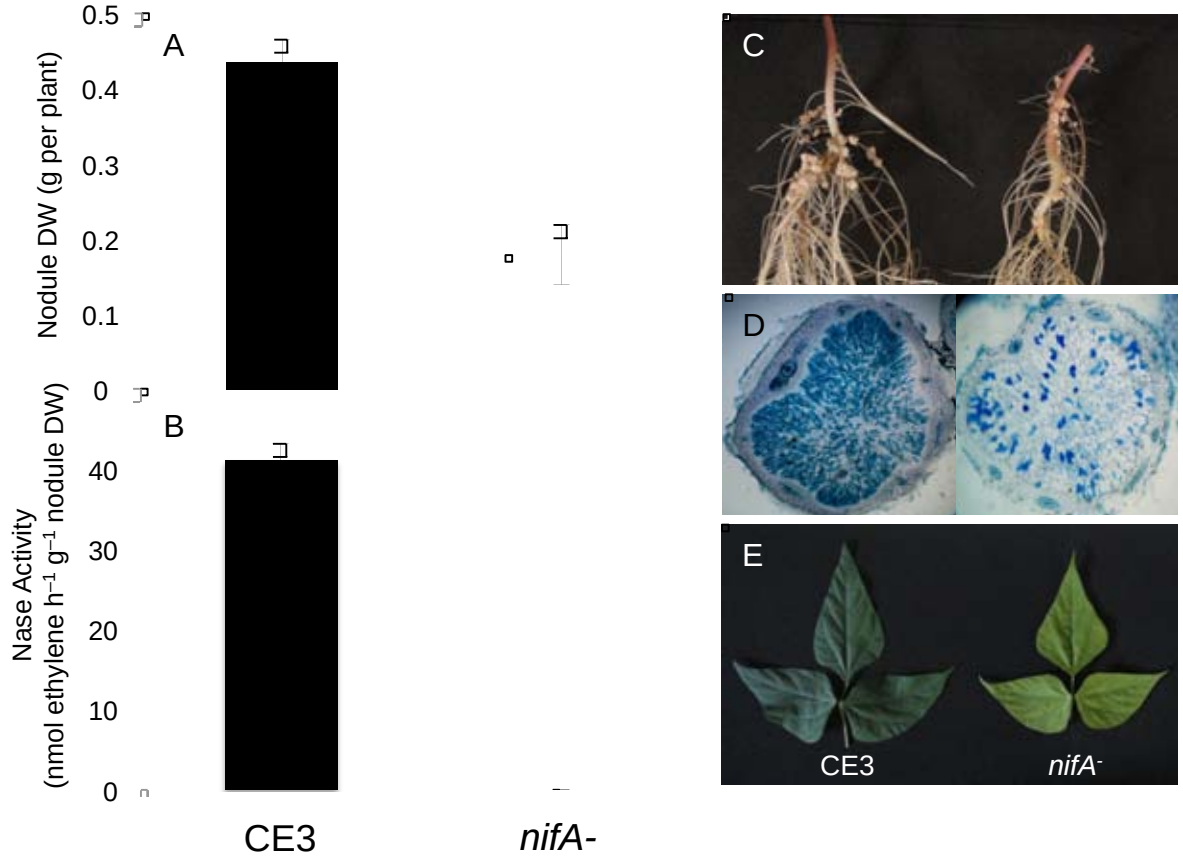
Zhu QH, Helliwell CA (2011) Regulation of flowering time and floral patterning by miR172. J Exp Bot **62:** 487–495

Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res **31:** 3406–3415

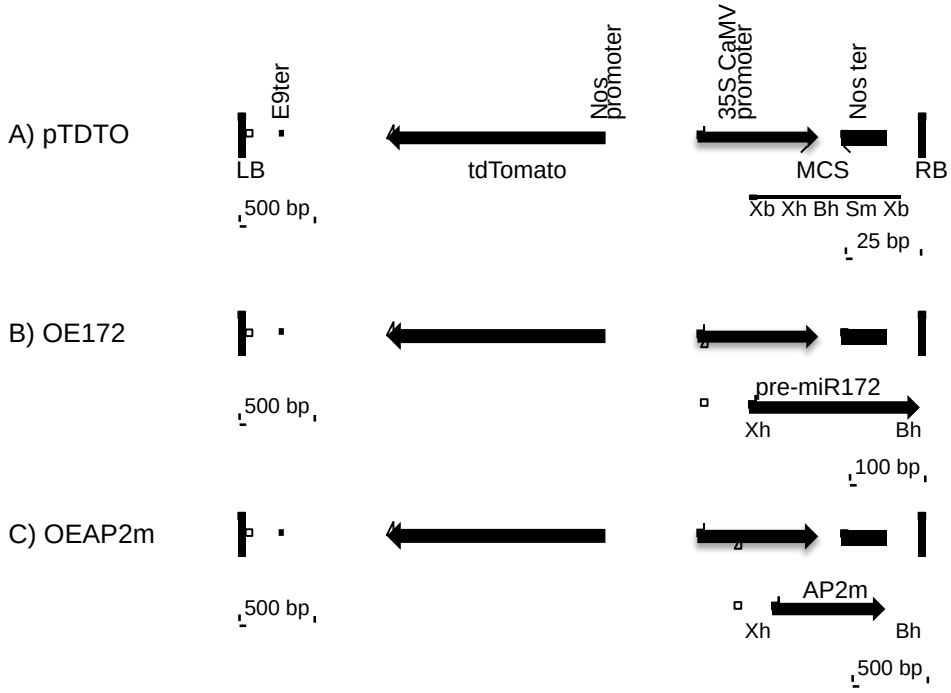
This work	Pelaez et al., 2012	Sequence	*P. vulgaris* genome mapping
miR172a (miR172a/e/f)	ath-miR172a	AGAAUCUUGAUGAUGCUGCA (20nt)	Chr. 5 pos. 35,820,612-631
miR172b	zma-miR172a	AGAAUCUUGAUGAUGCUGCAU (21nt)	Chr. 5 pos. 37,407,928-947
miR172c (miR172c/d)	ptc-miR172g	GGAAUCUUGAUGAUGCUGCAG (21nt)	Chr. 1 pos. 49,411,375-395
miR172d	ptc-miR172g	GGAAUCUUGAUGAUGCUGCAG (21nt)	Chr. 8 pos. 49,749,706-726
miR172e	ath-miR172a	AGAAUCUUGAUGAUGCUGCA (20nt)	Chr. 11 pos. 7,868,006-025
miR172f	ath-miR172a	AGAAUCUUGAUGAUGCUGCA (20nt)	Chr. 7 pos. 10,358,575-594



Supplemental Figure S1. miR172 isoforms encoded in the *Phaseolus vulgaris* genome and the most stable secondary structures predicted for their precursors. Data from analysis of the *P. vulgaris* genome sequence (Schmutz et al., 2014; www.phytozome.net V.1.0). The previous designation of each miR172 isoform according to Peláez et al. 2012 is provided. The most stable secondary structure predicted for each pre-miR172 using Mfold is shown, mature miR172 sequence are highlighted with red lines and yellow boxes.



Supplemental Figure S2. Symbiotic phenotype of common bean plants inoculated with *Rhizobium etli nifA*- mutant strain as compared to CE3 wild type strain. Determinations were done at 18 dpi. A, Nodules per plant (DW), n=10 plants. B, Nitrogenase activity (acetylene reduction assay) n=10 plants. C, Nodulation-competent zone of roots. D, Light micrographs of methylene blue-stained transverse section of nodules. E, Trifoliated leaves (first, oldest, trifolium)



D) miR172 cleavage site

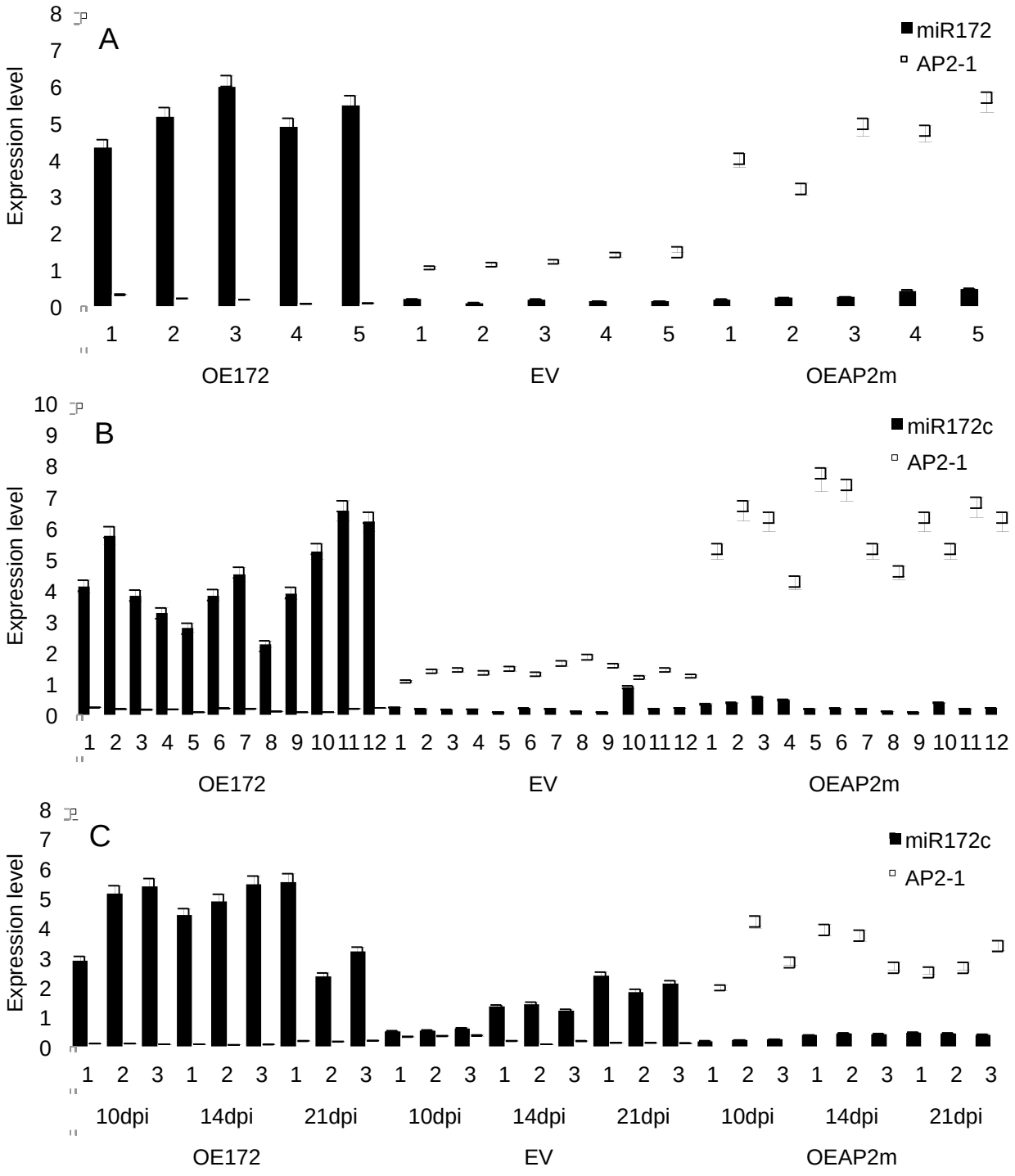
D.1. Mutated AP2-1

```
593    CT GCc* GCc ***agA TCt GGA TTC T     613
199       Ala Ala Arg Ser Gly Phe           204
```

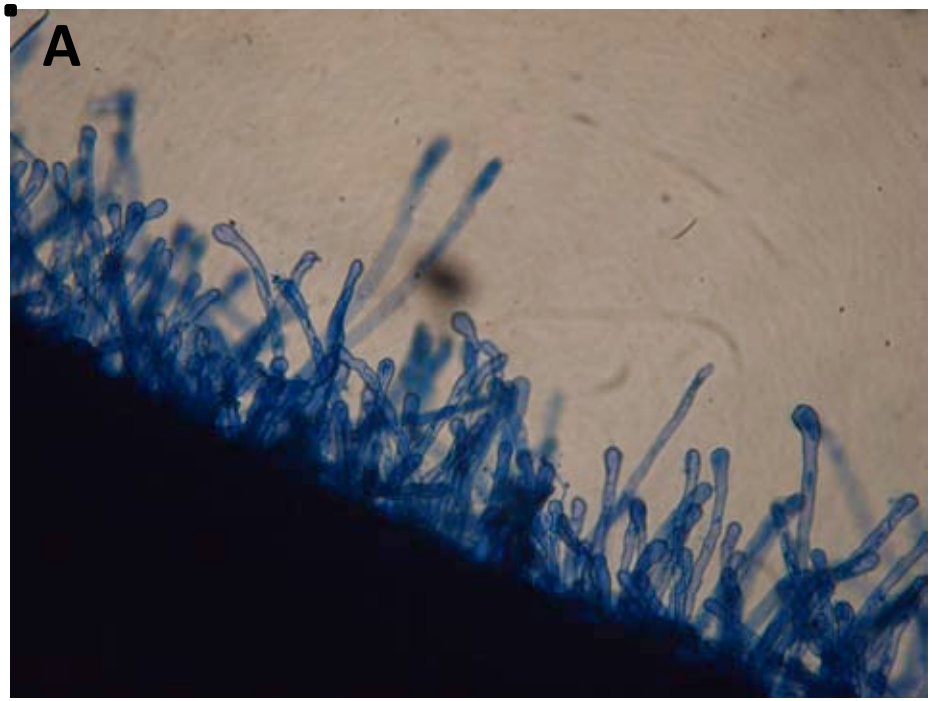
D.2. WT AP2-1

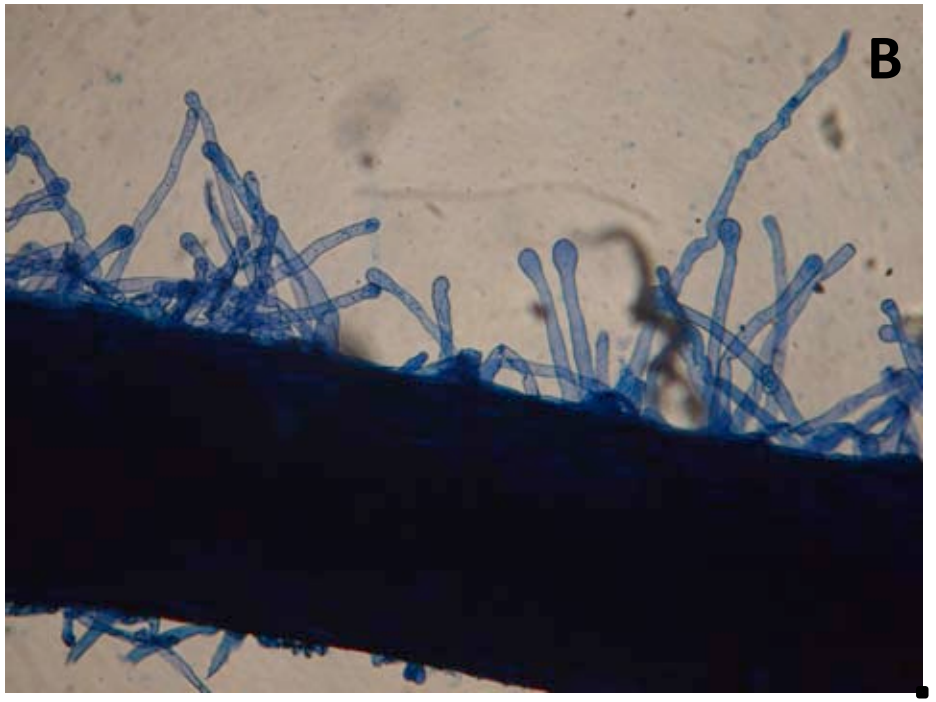
```
593    CT GCA GCA TCA TCA GGA TTC T     613
199       Ala Ala Ser Ser Gly Phe       204
```

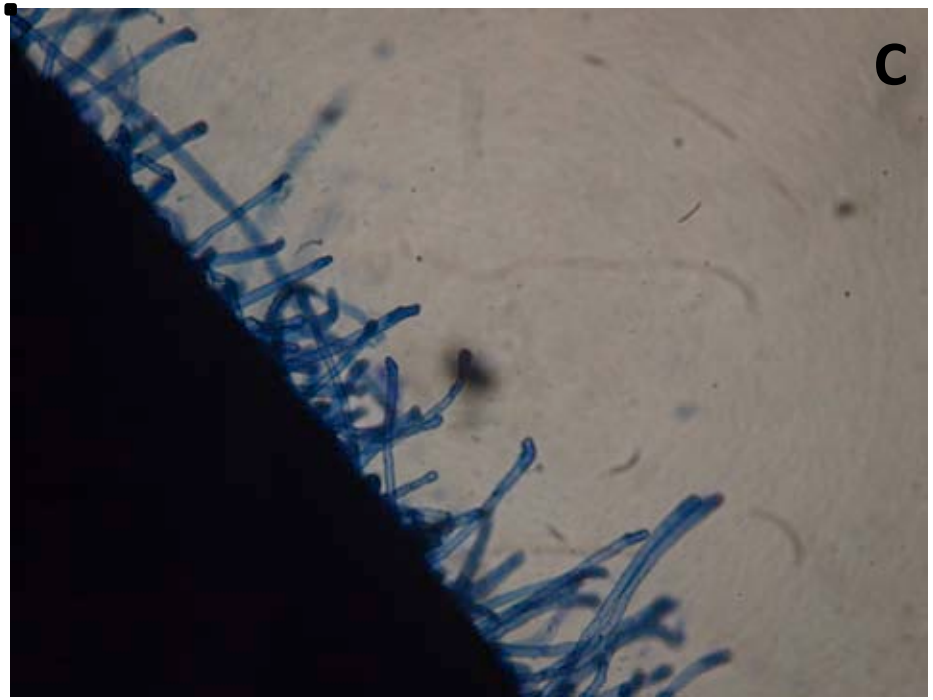
Supplemental Figure S3. Schematic representation of plasmids used for miR172c or *AP2-1* overexpression. (A) Plasmid vector pTDTO and its derivatives with (B) miR172c precursor (pre-miR172c) and (C) mutagenized *P. vulgaris AP2-1 (AP2m)* that is insensitive to miR172 cleavage. (D) miR172 cleavage site of *AP2-1.* Numbers correspond to nucleotides and aminoacids in the *AP2-1* coding region. * Indicates the nucleotides modified to generate a mutated miR172 cleavage site. *Bgl*II and *Pst*I restriction sites are underlined.



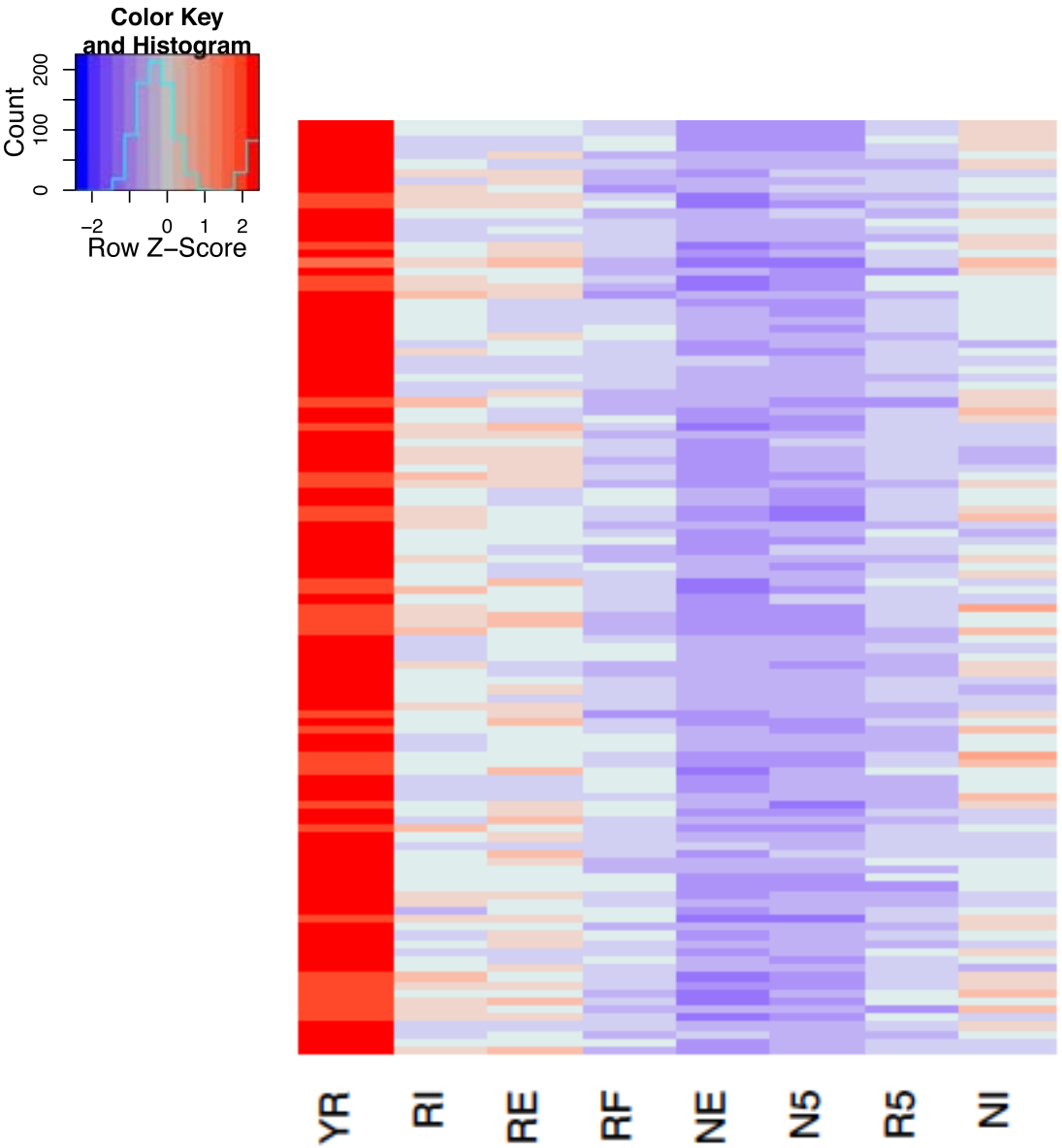
Supplemental Figure S4. Over-expression of miR172c and of *AP2m* in transgenic roots and nodules of composite bean plants. Expression analysis from transgenic roots (A,B) and nodules (C) of OE172, EV or OEAP2m composite plants was performed by qRT-PCR. Each bar (black = miR172c; white = *AP2-1*) represent the transcript levels from tissue of an individual composite plant. A, Roots from 5 plants with each construct watered with full-nutrient solution (+N). B, Nodule-detached roots from 12 *R. etli*-inoculated plants (21 dpi) with each construct C, Nodules detached from roots of 3 individual plantswith each construct, at different dpi. The values represent the average (± SD) from two technical replicates each.



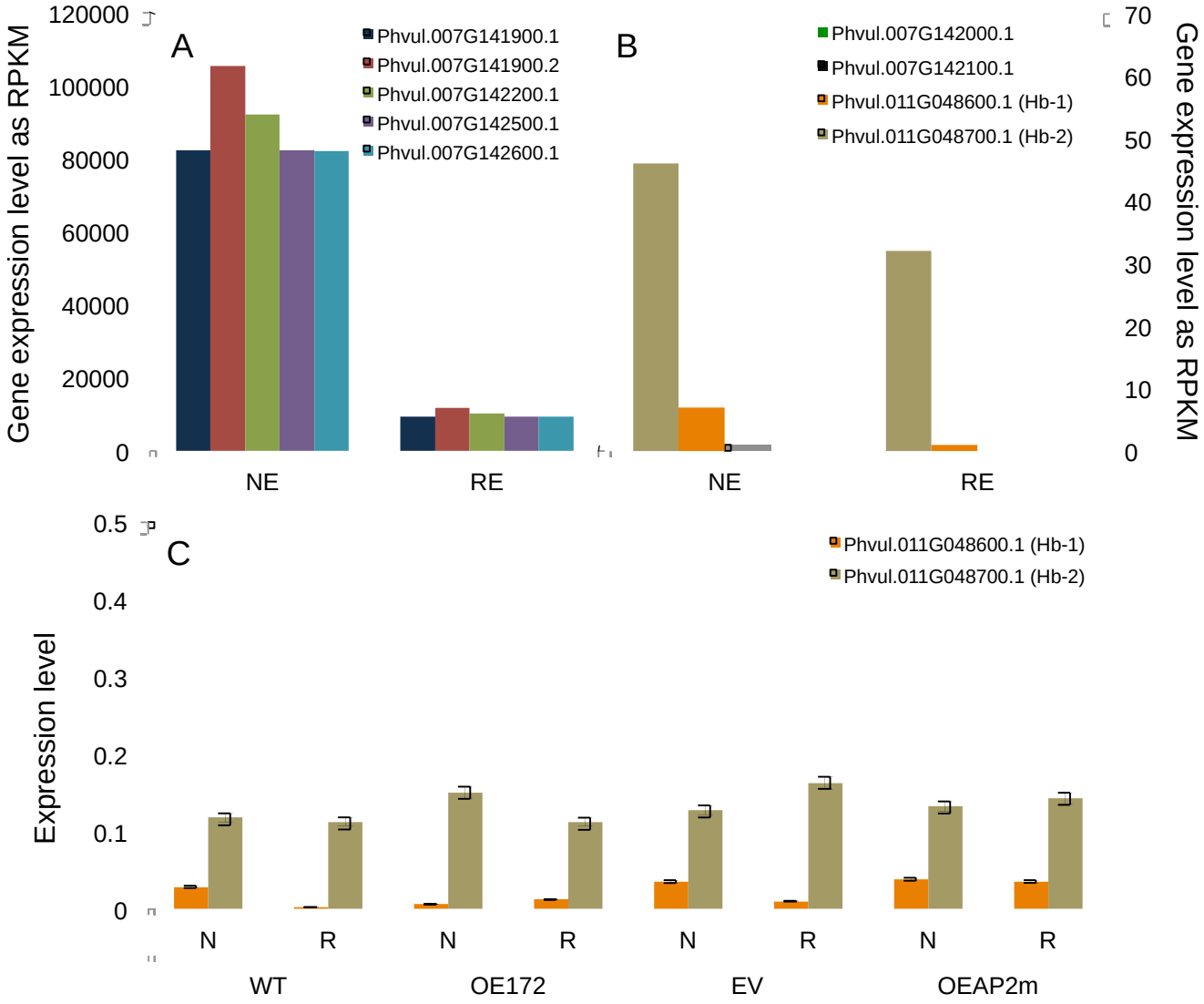




Supplemental Figure S5. miR172 and AP2 control the rhizobia infection in common bean roots.
Transgenic roots expressing OE172 (A),Empty Vector (B) or OEAP2m (C) were inoculated with *R. etli* C3. After 48 h post-inoculation, number of deformed root hairs (branched- and swollen) from the root responsive zone were quantified.



Supplemental Figure S6. Expression pattern of *AP2-1* co-expressed genes. Expression profile (as Z-scores: red = high, blue = low) of 144 root-enhanced genes listed in Supplemental Table S4. Tissue samples: YR=young roots, RI=ineffective nodule-detached roots (21 dpi), RE=effective nodules-detached roots (21 dpi), RF=roots from non-symbiotic plants grown in full-nutrient solution, NE=effective (21 dpi) nodules, N5=pre-fixing effective (5 dpi) nodules, R5=nodule-detached roots from 5dpi, NI= ineffective (21 dpi) nodules



Supplemental Figure S7. Expression analysis of *P. vulgaris* symbiotic (*Lb*) and non-symbiotic (*Hb*) hemoglobin genes. *Lb* (A) and *Hb* (B) gene expression levels as RPKM from Pv GEA data. NE: effective (21 days post inoculation, dpi) nodules, RE: effective nodule-detached roots (21 dpi) (O'Rourke et al., 2014). C, Expression of Phvul011G048600 and Phvul011G048700 *Hb* genes was determined by qRT-PCR in mature nodules (N) and nodule-detached roots (R) and in of *R. etli*-inoculated wild type and composite OE172, EV or OEAP2m plants. Values represent the average (± SD) from three replicate samples per time point.

Supplemental Table S1. Primer sequences for qRT-PCR

Gene Name	Gene ID	Forward Primer (5' - 3')	Reverse Primer (3' - 5')
miR172a	Chr. 1	AGAATCTTGATGATGCTGCA	
miR172b	Chr. 5	AGAATCTTGATGATGCTGCAT	
miR172c	Phvul.001G233200	GGAATCTTGATGATGCTGCAG	
miR156a	Chr. 9	TGACAGAAGAGAGTGAGCAC	
miR159	Chr. 4	TTTGGATTGAAGGGAGCTCTA	
UBC9	Phvul.006G110100	GCTCTCCATTTGCTCCCTGTT	TGAGCAATTTCAGGCACCAA
AP2-1	Phvul.005G138300	CAGCTACCTTTCCGCCAAATGC	TAGGCTGGGATGGTGTCTGCAG
AP2	Phvul.011G071100	CGGAAAATTCACCTGGCGTC	TCCGATCGAAAGCAACCGAA
AP2	Phvul.001G174400	ATCGCGGCGTCACTTTCTAT	GCAGCTCGATCATAAGCCCT
AP2	Phvul.003G241900	AAAGCACTGGATTCCCGAGG	TGCAGCTTCAATCTCGGTGT
AP2	Phvul.002G016900	AACGGAGGCCGTATTGGAAG	GCCAACCTAGGGAGGGTCTA
AP2	Phvul.007G240200	ACTGAAGGCAACAAGGAGCA	CTCCCACAACCCCGATTTGA
SPL	Phvul.009G165100	TGCAGATGCTAGGTTGCACA	GTGGAGGGATGGTTACCGTG
NOD55	Phvul.003G155600	AATAACCAGTGGCCAACATCA	AGTGCTTCCTAAACGCAAACA
NF-YA1 (HAP2)	Phvul.001G196800	TACTTTGGCAATCCATCCTTG	AGACAGTTCGGTGCAGAAAGA
NSP2	Phvul.009G122700	GACGGTTATCGGGTAGAGGAG	CGGAGGAAGAAGAAGTCCAAA
CYCLOPS	Phvul.002G128600	TCCTTACCACATTCTGCTGAGA	CCAAGAGATTCCAGAGGTTCA
NIN	Phvul.009G115800	GGGAGAAGAGGCGTACGAAG	GTTGTGGGACACACTCCGAT
ENOD40	Phvul.002G064200	GGGTCCTTACCCCTCACACT	TGTAGCCAAAGCCTCTCATCC
FLOT2	Phvul.009G090700	GGAACCCTGTCAGACAAAACA	TTCACGAATCCAAAACAACC
PEPC	Phvul.005G066400	AAGTGAGTATGCCCCTGGTTT	GAAAGGGAAGATGGGTGAAAG
Cystein-Proteinase CP	Phvul.003G240800	CCTCTGGCAGGGCTTTGCAACT	GGTGCTTCTCACGTTGCGCT
Aminocyclopropanecarboxylate oxidase	Phvul.002G326600	GGAGCACCGTGTGATTGCTA	CCAACAATGGTGGTGCAGGA
Serine/threonine protein kinase	Phvul.007G049500	GCGGTCTCCTTCACGCTTAT	GCTGTTGGCTGTGTAGTTGC
Serine/threonine protein kinase	Phvul.007G049000	CTGGTGGAACACAGCAAAGG	GCAGCATACTTACGACGGGA
Glycogen synthase kinase-3	Phvul.006G174500	ATACTCCCCAAGTCTCCGGT	CATTTGGCAGGCGAGCATTG
Serine/threonine protein kinase	Phvul.011G169600	AGCCTTGCGCAAACCATTTT	CACGTGCAATCCCAATGCAA
Serine/threonine protein kinase	Phvul.008G263900	GGGGAGCGTCTAGACATAGC	TGCCACTTTGGCCTTTAGGT
Hb-1	Phvul.011G048600	TGTGGTCCCCGGAAATGAAG	GCACAGACCCAAACACAACC
Hb-2	Phvul.011G048700	ATTCTGCAGAGTTGGGCCTC	TGCTCCAAAGGAACGGTTGA
RIC1	Phvul.005G096900	TATTGCGGACAGCCATCATCA	TCCCTCTGGTGCGAGTCTAT
NIC1	Phvul.005G097000	AGAAGACCCAACTCGAAGGT	TCCTCCGGGGCTTAGTCTTT

Supplemental Table S2. TF binding sites (TFBS) identified in the 5' promoter region of each *MIR172* gene. The number of TFBS statistically represented ($P < 0.05$) in the 1,000 bp region upstream of the transcription-start site of *MIR172a* and *MIR172c* genes and in the 1,500 bp region upstream of the precursor with most stable secondary structure of *MIR172b, d, e* and *f* genes (Supplemental Fig. S1) are indicated.

	miR172a	miR172b	miR172c	miR172d	miR172e	miR172f	
AG_01					2		
AG_03					2		
AG_Q2				2	2	2	
AGL15_01				5			2
AGL3_02				4			
AGL3_03				4			
ARF_Q2					3		
ATHB1_01							2
ATHB9_01					2		
CPRF2_01					2		
CPRF2_Q2					2		
CPRF3_01					2		
CPRF3_Q2					2		
DOF_Q2		2					
DOF1_01							2
GT1_Q6							2
HAHB4_01			3				3
HBP1A_Q2					2		
HBPA1_Q6_01					2		
HDG9_01					2		
MADSA_Q2						2	
MADSB_Q2			2				
ML1_01					2		
MYBPH3_02		2					
NAC691_01						2	
O2_02					2		
PBF_01				2			
PDF2_01					3		
PEND_01						2	
SBF1_01		2	3		4		4
SED_Q2		2					
STF1_01					2		
TAF1_Q2					2		
TGA1A_Q2					2		
TGA2_01		2			3		

Supplemental Table S3. *Nitrogenase activity and expression analysis of marker genes for nodule development.*

Nitrogenase (Nase) activity assayed by ARA, and transcript level for each marker gene assayed by qRT-PCR, were determined in nodules harvested at different dpi from inoculated common bean plants grown under different treatments, as indicated. The selected marker genes are: PvENOD55: Early nodulin 55 (Phvul.003G155600) for early (immature) nodules, PvPEPc: Phosphoenolpyruvate carboxylase (Phvul.005G066400) for mature nodules and PvCP: Cystein proteinase (Phvul.003G240800) for late (senescent) nodules. Values are expressed relative to the highest Nase activity or expression value for each gene (100%) in effective nodules, these represent the average (±SD) from three biological replicates and two technical replicates each.

Samples	**Nase Activity (%)**	**Relative expression (%)**		
		ENOD55	**PEPc**	**CP**
Effective nodules elicited by *R. etli* CE3 wt strain				
13dpi	19.6	**100**	3.6	0.03
18dpi	**100**	5.6	**100**	0.04
25dpi	49.0	3.9	25.1	9.7
35dpi	11.2	1.9	3.8	**100**
Ineffective nodules elicited by *R.etli nifA*- mutant strain				
13dpi	0.0	177.5	3.4	0.27
18dpi	0.0	100.8	2.5	43.3
25dpi	0.0	56.7	1.7	148.7
35dpi	0.0	16.2	1.3	71.5
NO_3 treated effective nodules				
18dpi –NO_3 (Control)	**100**	5.6	**100**	0.04
19dpi + NO_3 (1 day post treatment)	28.5	0.36	67.8	11.1
21dpi + NO_3 (3 day post treatment)	11.7	0.56	0.58	58.8

Supplemental Table S4. *Expression analysis of marker genes for nodule development in transgenic nodules of OE172, EV or OEAP2m plants.*

The expression of the marker genes indicated in Supplemental Table S1 was determined. ND: Not determined. Values are expressed relative to the highest expression value for ENOD55 or PEPc (100%) in effective nodules of EV plants; these represent the average (± SD) from three biological replicates and two technical replicates each.

Samples	**Expression level**								
	ENOD55			**PEPc**			**CP**		
Transgenic effective nodules (-N)	**OE172**	**EV**	**OEAP2m**	**OE172**	**EV**	**OEAP2m**	**OE172**	**EV**	**OEAP2m**
10dpi	63	**100**	276	143	17	13	ND	ND	ND
14dpi	47	92	211	229	68	32	ND	ND	ND
21dpi	37	41	156	339	**100**	52	2	10	48
30dpi	ND	ND	ND	ND	ND	ND	32	100	116
NO_3 treated transgenic nodules									
1mM									
14dpi	165	0	0	96	0	0			
21dpi	93	261	0	468	62	0			

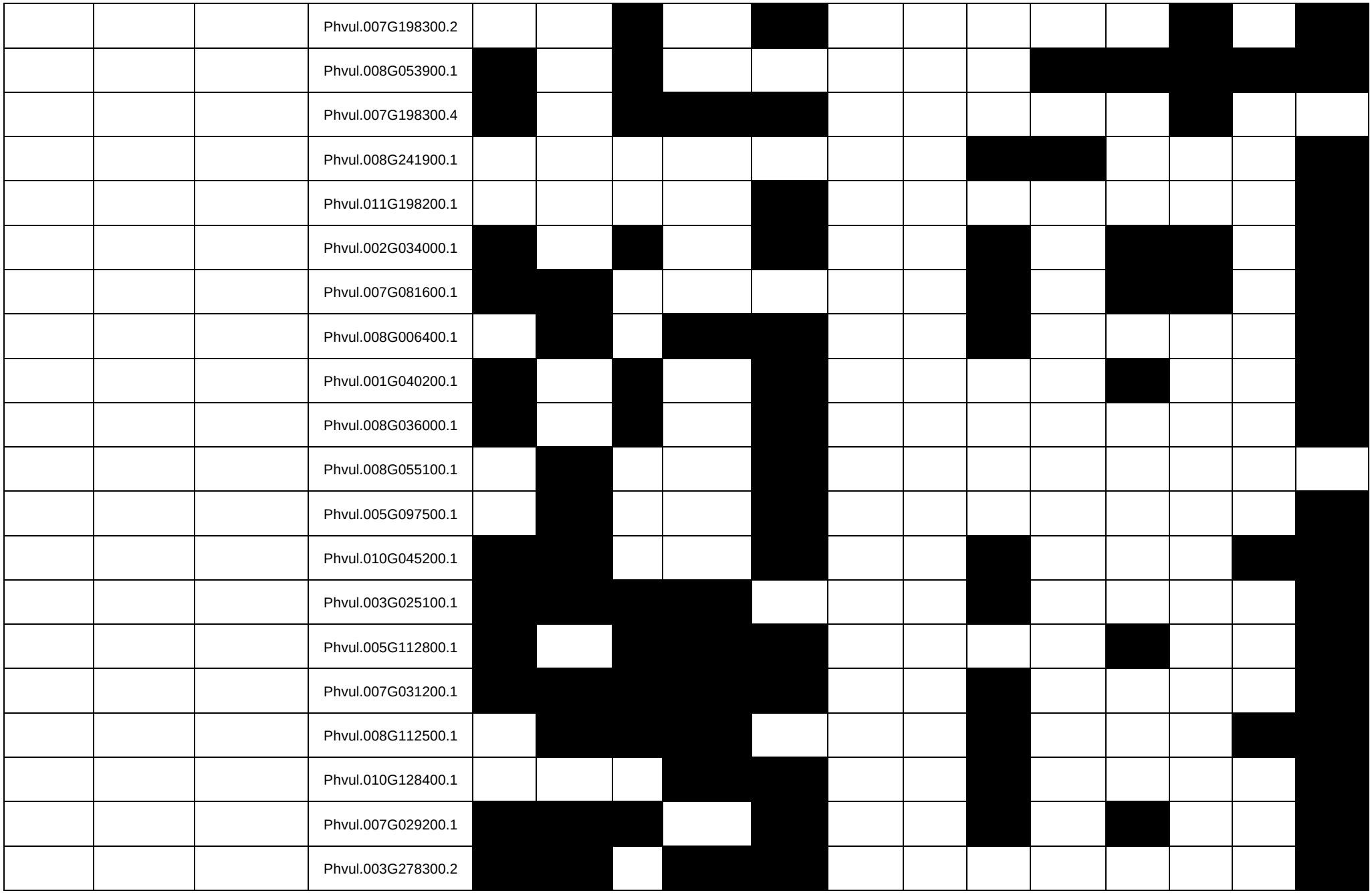
Supplemental Table S5. *AP2-1* co-expressesed genes: assigned GO categories and identified TF binding sites (TFBS). No GO category could be assigned to the genes indicated in the last rows. TFBS statistically represented ($P < 0.05$) in the 1,000 bp region upstream of the ATG-start codon of each gene are marked with a black box.

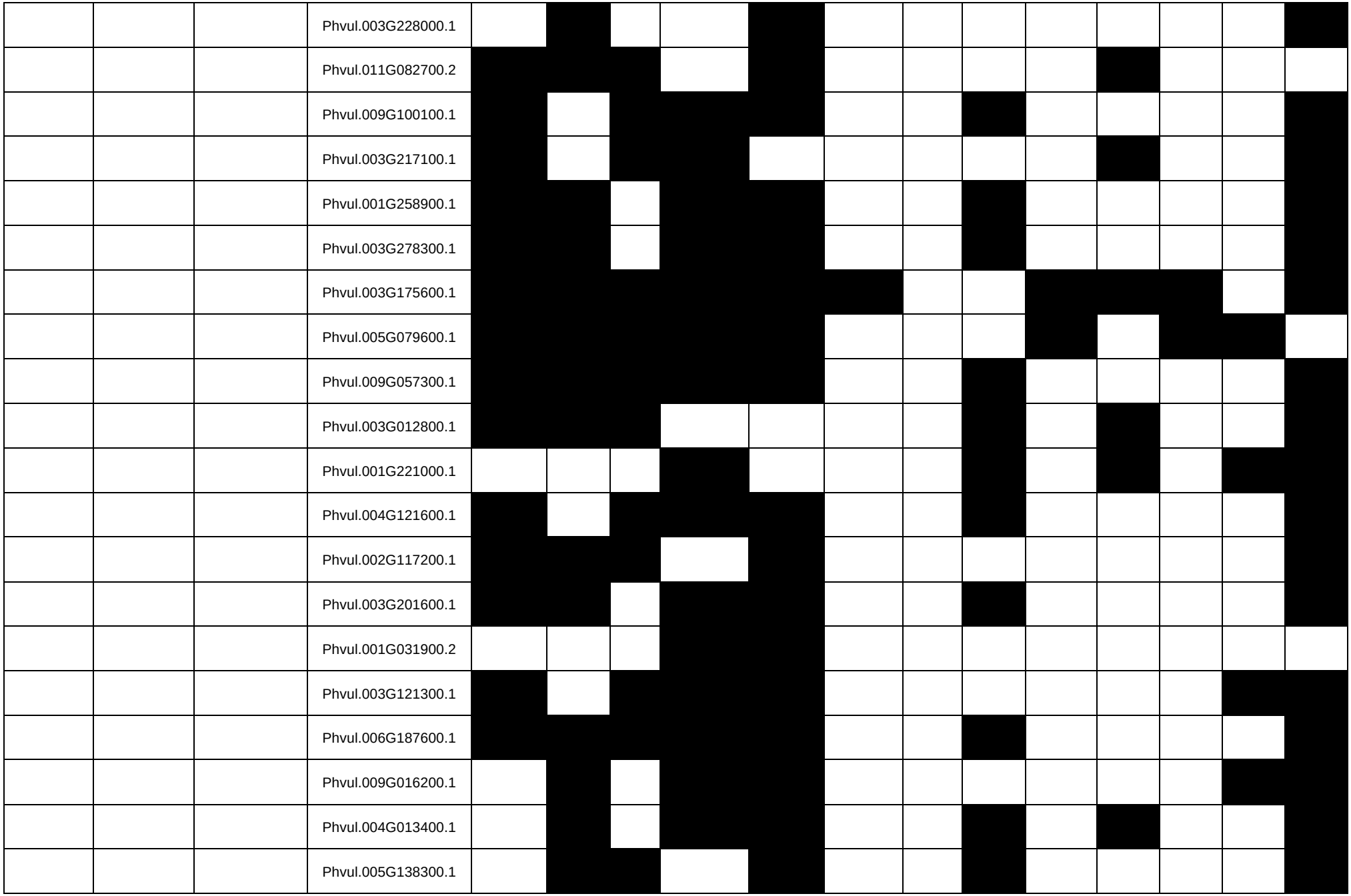
				TFBS (P-value)												
GO	**Molecular function**	**P-value**	**Genes ID**	**DREB1B (0)**	**WRKY11 (0)**	**ERF2 (0.001)**	**TGA1A (0.004)**	**PBF (0.008)**	**ATHB9 (0.009)**	**HMGIY (0.01)**	**BPC (0.015)**	**BZIP9 (0.018)**	**ABI4 (0.019)**	**ABF (0.02)**	**CBT (0.043)**	**DOF (0.047)**
GO:0004672	Protein kinase activity	0.006146478	Phvul.001G022300.2	■		■	■	■			■		■			■
			Phvul.001G022300.1	■		■	■	■			■		■			■
			Phvul.001G014400.1	■	■			■			■		■	■		■
			Phvul.002G326600.1	■	■						■					■
			Phvul.009G050100.1	■	■	■	■	■			■	■	■			
			Phvul.002G196200.1		■		■	■			■					■
			Phvul.007G049500.1		■	■							■			
			Phvul.011G169300.1	■	■	■	■	■			■		■	■		■
			Phvul.008G190300.1		■											
			Phvul.007G049000.1	■	■		■	■								■
			Phvul.002G289000.1	■	■			■			■					■
			Phvul.006G174500.1	■	■		■	■			■	■	■	■		■
			Phvul.008G233400.1	■	■		■	■								■
			Phvul.008G007900.2				■	■				■				■
			Phvul.008G263900.2	■	■	■	■	■			■	■	■	■		
			Phvul.004G173300.1	■				■			■					■
			Phvul.011G169600.1	■	■		■	■			■					

			Phvul.006G187700.1														
			Phvul.011G194400.1														
			Phvul.007G049400.1														
GO:0008703	5-amino-6-(5-phosphoribosylamino)uracil reductase activity	0.0162696	Phvul.008G053900.2														
GO:0005516	Calmodulin binding	0.018911169	Phvul.009G254500.1														
			Phvul.009G254500.2														
GO:0005471	ATP:ADP antiporter activity	0.024305769	Phvul.001G074700.1														
GO:0004351	Glutamate decarboxylase activity	0.055805138	Phvul.006G087600.1														
GO:0004435	Phosphatidylinositol phospholipase C activity	0.055805138	Phvul.001G243300.1														
GO:0005524	ATP binding	0.088476713	Phvul.005G173600.1														
			Phvul.002G173000.1														
			Phvul.007G209600.1														
			Phvul.005G173700.1														
			Phvul.002G034000.2														
			Phvul.009G233700.1														
GO:0042578	Phosphoric ester hydrolase activity	0.108523046	Phvul.010G125000.1														
GO:0008378	Galactosyltransferase activity	0.205365153	Phvul.003G146700.1														
GO:0015662	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	0.211869261	Phvul.009G234600.1														
GO:0016762	Xyloglucan:xyloglucosyl transferase activity	0.231066764	Phvul.003G137600.1														
GO:0008080	N-acetyltransferase activity	0.274077828	Phvul.002G044100.1														

GO:0016760	Cellulose synthase (UDP-forming) activity	0.280024432	Phvul.006G058700.1													
GO:0051287	NAD binding	0.297576174	Phvul.005G166400.1													
GO:0005543	Phospholipid binding	0.303332015	Phvul.007G003700.1													
GO:0016757	Transferase activity, transferring glycosyl groups	0.41396646	Phvul.009G177200.1													
GO:0022857	Transmembrane transporter activity	0.595811179	Phvul.007G003700.2													
GO:0005529	Sugar binding	0.627902654	Phvul.005G065700.1													
GO:0050660	Flavin adenine dinucleotide binding	0.692447561	Phvul.011G098500.1													
GO:0016706	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors	0.700003165	Phvul.002G326600.2													
GO:0003824	Catalytic activity	0.888175173	Phvul.011G064200.1													
			Phvul.008G018200.1													
			Phvul.011G082700.1													
GO:0043565	Sequence-specific DNA binding	0.900994367	Phvul.008G263900.1													
			Phvul.001G060200.1													
			Phvul.008G251800.1													
GO:0003700	Sequence-specific DNA binding transcription factor activity	0.903453967	Phvul.005G138300.2													
GO:0016787	Hydrolase activity	0.917660822	Phvul.010G121500.1													
GO:0003677	DNA binding	0.958589007	Phvul.002G206300.1													
			Phvul.002G273100.1													
			Phvul.010G115500.1													
			Phvul.009G156300.1													

GO:0008270	Zinc ion binding	0.991938616	Phvul.007G253000.1	
GO:0005515	Protein binding	0.997263018	Phvul.007G079900.1	
			Phvul.002G241400.1	
			Phvul.008G196400.1	
GO	**Biological Processes**	**P-value**	**Genes ID**	**TFBS (P-value)**
GO:0005985	Sucrose metabolic process	0.070676933	Phvul.001G209600.1	
GO:0009607	Response to biotic stimulus	0.318682263	Phvul.003G109000.1	
GO:0006886	Intracellular protein transport	0.623234886	Phvul.009G132300.1	
GO:0055085	Transmembrane transport	0.869649335	Phvul.006G060800.2	
GO:0055114	Oxidation-reduction process	0.992475524	Phvul.002G209200.1	
			Phvul.011G168100.1	
			Phvul.007G002500.1	
			Phvul.008G199900.1	
			Phvul.006G193600.1	
			Phvul.007G032300.1	
			Phvul.007G198300.1	
			Phvul.007G198300.3	
			Phvul.010G021200.1	


Phvul.007G198300.2
Phvul.008G053900.1
Phvul.007G198300.4
Phvul.008G241900.1
Phvul.011G198200.1
Phvul.002G034000.1
Phvul.007G081600.1
Phvul.008G006400.1
Phvul.001G040200.1
Phvul.008G036000.1
Phvul.008G055100.1
Phvul.005G097500.1
Phvul.010G045200.1
Phvul.003G025100.1
Phvul.005G112800.1
Phvul.007G031200.1
Phvul.008G112500.1
Phvul.010G128400.1
Phvul.007G029200.1
Phvul.003G278300.2


Phvul.003G228000.1
Phvul.011G082700.2
Phvul.009G100100.1
Phvul.003G217100.1
Phvul.001G258900.1
Phvul.003G278300.1
Phvul.003G175600.1
Phvul.005G079600.1
Phvul.009G057300.1
Phvul.003G012800.1
Phvul.001G221000.1
Phvul.004G121600.1
Phvul.002G117200.1
Phvul.003G201600.1
Phvul.001G031900.2
Phvul.003G121300.1
Phvul.006G187600.1
Phvul.009G016200.1
Phvul.004G013400.1
Phvul.005G138300.1

CAPITULO V Exploración del papel regulatorio del nodo miR172-*AP2* de *Medicago truncatula* en la simbiosis con *Sinorhizobium meliloti*

Como ya se mencionó anteriormente, miR172 y AP2 poseen un papel regulatorio en la nodulación y FSN en frijol y en soya (Yan et al., 2013; Wang et al., 2014; Nova-Franco et al., 2015). Debido a estos antecedentes se planteó un proyecto para explorar la función de miR172 en la nodulación y FSN de *Medicago truncatula*.

Medicago truncatula comúnmente llamada simplemente Medicago, posee diferentes características que la han hecho una excelente leguminosa modelo para la investigación genética y genómica. Es miembro de la subfamilia Papilionoideae de las leguminosas y es filogenéticamente cercana a *Medicago sativa* que comúnmente se conoce como alfalfa y es la leguminosas que más se utilizan como forraje. Medicago es diploide y posee un genoma relativamente pequeño (su tamaño haploide es aproximadamente de 550 Mbp). Medicago es autógama y produce una gran cantidad de semillas para una planta que se considera pequeña. Es una leguminosa relativamente fácil de transformar genéticamente para generar transformantes estables por lo cual se pueden llevar acabo estudios de genética reversa en plantas (Cosson et al., 2006). Por último en la investigación de Medicago principalmente se utilizan dos ecotipos. El ecotipo Jemalong A17 de *M. truncatula,* originalmente aislado de Austrialia y que fue utilizado en el proyecto de secuenciación del genoma de Medicago para generar el genoma de referencia (Town 2006); y el ecotipo R108, derivado del ecotipo R108-1 por medio de regeneración *in vivo* el cual actualmente se utiliza en herramientas genéticas como las líneas mutantes y transformantes estables de Medicago. Los ecotipos A17 y R108 son diferentes tanto fenotípica como genotípicamente (Hoffmann et al., 1997).

Debido a que se considera una planta modelo se han desarrollado diferentes herramientas para su estudio, las cuales incluyen mapas genéticos, diferentes poblaciones de mutantes (incluyendo mutantes por EMS (Etil Metano Sulfonato),

deleciones por “fast-neutron y inserciones de transposones), así como también herramientas y protocolos para el estudio de transcriptómica, proteómica y metabolómica.

Una de las instituciones que ha desarrollado y tiene acceso a diferentes colecciones y herramientas es The Samuel Robert’s Noble Foundation (Ardmore, OK, EUA) en donde realice una estancia de investigación durante mi Doctorado en el grupo del Dr. Michael Udvardi (Senior Vice President & Plant Biology Division Director). Durante esta estancia inicié el proyecto sobre el papel de miR172/AP2 en Medicago.

La población de mutantes de Medicago por medio de inserción de Tnt1 que posee The Noble Foundation, es la colección mas grande de mutantes por inserción de DNA que existe actualmente en leguminosas. Se estableció en el genotipo R108 por medio de inserción del transposon Tnt1 mediante transformación con *Agrobaterium tumefaciens* (*d’Erfurt et al., 2003*). A la fecha se tienen casi 20,000 líneas independientes que contienen inserciones en diferentes loci y las cuales han sido generadas por embriogénesis somática de explantes de hoja.

Otra de las herramientas mas reciente que se ha desarrollado dentro de The Noble Foundation para el estudio de genómica funcional de Medicago es el *Medicago Gene Expression Atlas*, el cual incluye expresión génica cuantitativa para la mayoría de sus genes durante diferentes estadios de el desarrollo de la planta así como genes que responden a diferentes estímulos ambientales como a la micorrizas, a la simbiosis con *Rhizobium* y la fijación simbiótica de nitrógeno (*Benedito et al., 2008*)

En mi proyecto de doctorado se demostró que el miR172 y su gene blanco *AP2* juegan un papel importante en el desarrollo del nódulo y en la FSN en la simbiosis frijol-*Rhizobium etli*. Sin embargo aún no se cuenta con la caracterización del nodo de miR172-*AP2* en los nódulos indeterminados de Medicago. Actualmente lo que se sabe acerca de miR172 en *M. truncatula* es lo reportado por *Lelandais-Briere et al.* (2009), en donde se demuestra que su expresión es más abundante en nódulos maduros y es específica de la

zona del meristemo. Además se ha demostrado que en *M. truncatula* que el gene blanco de miR172 pertenece a la familia de APETALA2 (*Jagadeeswaran et al., 2009*). Debido a lo anterior este proyecto planteó definir el papel del nodo miR172-AP2 en el desarrollo del nódulo y en la FSN de Medicago utilizando las tecnologías y herramientas que posee la The Samuel Robert's Noble Foundation.

Recientemente se publicaron dos artículos en soya, en donde se observa un efecto positivo en la sobreexpresión de miR172 en la nodulación (*Yan et al., 2013; Want et al., 2014*). Además en mi proyecto de doctorado, en frijol se observa el mismo efecto. La sobreexpresión de miR172 y la consecuente disminución de la expresión de su gene blanco AP2 aumenta el número de nódulos y la actividad de nitrogenasa (Capitulo IV; *Nova-Franco et al., 2015*).

Con base en lo anterior, la hipótesis de trabajo para el proyecto de Medicago es que tanto una transformante estable que sobreexprese el miR172 como una mutante nula en AP2 tendrán un fenotipo simbiótico positivo. Los resultados de este proyecto obtenidos hasta ahora se describen a continuación.

En este proyecto se planteó sobreexpresar el gene precursor de miR172 de *M. truncatula* en una transformante estable de Medicago. Para lo anterior se buscaron las diferentes isoformas de miR172 de Medicago en miRBase (www.mirbase.org). Se encontraron 4 isoformas (miR172a-miR172d) de las cuales solo 3 isoformas son diferentes entre si: miR172a, miR172b y miR172d (Tabla 1). Éstas se mapearon en el genoma de *M. truncatula* A17 (www.phytozome.net) y se predijo la estructura secundaria más estable de tallo y asa por medio mFold (http://mfold.rna.albany.edu) para dos de ellas: miR172a y miR172d. miR172a se encuentra dentro del cromosoma 5 y miR172d esta dentro de un contig de 2KB (contig_53419) (Fig. 15). De la isoforma miR172b no se encontró su precursor en el genoma debido a que la secuencia se encuentra dentro de un contig de 200pb, el cual es muy pequeño para su análisis.

Tabla 1. Isoformas de miR172 en *Medicago truncatula*		
Isoforma de miR172	Secuencia	Tamaño
miR172a	AGAATCCTGATGATGCTGCAG	21nt
miR172b	AGAATCTTGATGATGCTGCAT	21nt
miR172d	AGAATCTTGATGATGCTGCA	20nt

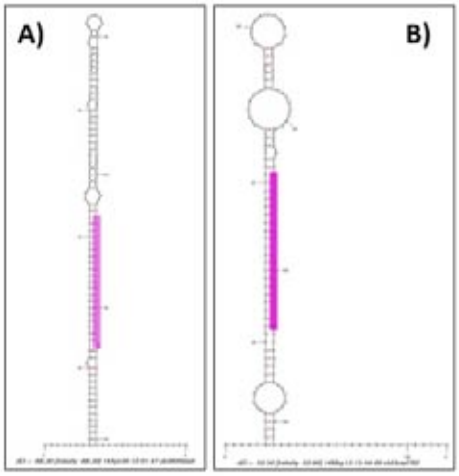


Figura 15. Estructura secundaria mas estable de los precursores de miR172 en el genoma *M. truncatula*. Se muestra la estructura mas estable predicha para dos isoformas de miR172a (A) y miR172d (B) utilizando mFold. La secuencia madura de miR172 se muestra en rosa.

Se analizó la expresión las tres isoformas de miR172 en raíces inoculadas con *Sinorhizobium meliloti* 1021 (0dpi – 8dpi). Los nódulos comienzan a desarrollarse a partir de los 10dpi por lo que se determinó la expresión en nódulos de (10dpi – 21dpi) los cuales incluyen tanto nódulos inmaduros como maduros. Se analizó la expresión por medio de qRT-PCR utilizando la secuencia de cada miR172 maduro como oligo 5'. Y se encontró que las tres isoformas solo se expresan en nódulos de 10dpi (Fig. 16).

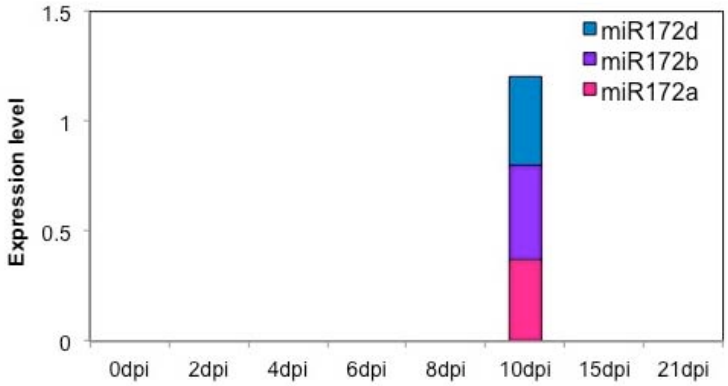


Figura 16. Análisis de la expresión de las tres isoformas de miR172 de *M. truncatula*. Se analizó la expresión de las tres isoformas de miR172 en el desarrollo del nódulo a los dpi indicados por medio de qRT-PCR. El nivel de expresión basa en el valor de C_t calculado utilizando al gene U6 snRNA como constitutivo. Los valores representan el promedio ± SD de tres replicas biológicas.

M. truncatula posee nódulos indeterminados, que tienen una zona meristemática persistente. *Lelandais-Briere et al.* (2009) reportó por hibridación in situ que miR172 se expresa principalmente en la zona del meristemo. Por esa razón se analizó la expresión tanto de miR172 y de *AP2-1* en las diferentes zonas de los nódulos maduros de 21 dpi de *M. truncatula*. Lo que se observó fue una expresión muy baja de *AP2-1* en todas las zonas de nódulo mientras que la isoforma miR172d es la que más se expresa en la solo en la zona meristemática del nódulo (Fig. 17).

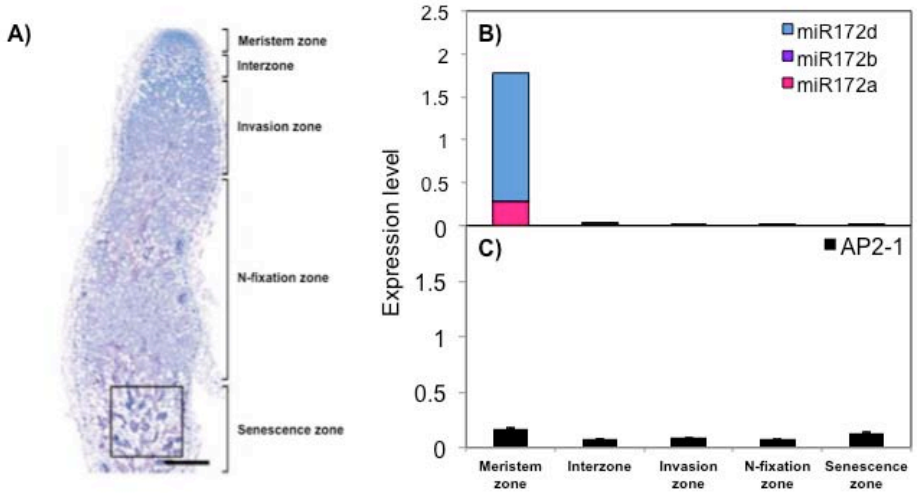


Figura 17. Análisis de la expresión de las tres isoformas de miR172 y AP2-1 en las diferentes zonas del nódulo de *M. truncatula*. Se analizó en las diferentes zonas de nódulos de 21dpi de Medicago (A), la expresión de las tres isoformas de miR172 (B) y de AP2-1 (C) por medio de qRT-PCR. El nivel de expresión basa en el valor de C_t calculado utilizando a los genes U6 snRNA y EF1 como constitutivos de referencia de miR172 y AP2-1 respectivamente. Los valores representan el promedio ± SD de tres replicas biológicas.

Lo anterior confirma los resultados ya reportados acerca de la expresión especifica de miR172 en la zona meristemática del nódulo (*Lelandais-Briere et al., 2009*).

En este proyecto se planteó obtener transformantes estables de Medicago que sobreexpresaran a miR172, lo cual es posible realizarse en el genotipo R108. Para ello, primero se buscaron los precursores de las isoformas de miR172 en las bases de datos de secuencias genómicas parciales de este ecotipo que se tienen disponibles en la The Noble Foundation y la isoforma miR172a fue la única isoforma que se pudo mapear. Por ello se clonó este gene en un vector con un promotor constitutivo 35S y actualmente se están generando transformantes estables de Medicago que sobreexpresen el miR172a

para realizar el análisis del fenotipo simbiótico y poder proponer una función de miR172 en los nódulos indeterminados de Medicago.

Además del análisis de miR172 en Medicago, también se analizaron los datos sobre la expresión de los genes *AP2* de *M. truncatula* en distintas condiciones tanto en el *Medicago Gene Expression Atlas* (www.mtgea.noble.org) como en datos no publicados de la expresión por qRT-PCR (Sinharoy S. Comunicación Personal). Se encontró que, de los 60 genes *AP2* reportados para Medicago, 10 genes presentan una expresión elevada en raíces con respecto a nódulos (perfil de expresión esperado). Por análisis de la secuencia de los AP2 se predijeron sitios de unión a miR172 en solamente 2 (de 10) genes *AP2* (Medtr2g093060 y Medtr5g016810). Se analizó la expresión de estos dos genes *AP2* predichos como blancos del miR172, por medio de qRT-PCR en raíces inoculadas con *S. meliloti* 1021 a diferentes días post inoculación (0dpi, 2dpi, 4dpi, 6dpi, 8dpi) y en nódulos (10dpi, 15dpi y 21dpi). Los datos indican que solamente el gene Medtr2g093060 presenta la expresión diferencial que esperábamos a diferencia del otro gene analizado y se observa que Medtr2g093060 tiene un nivel elevado en raíces inoculadas de 0dpi a 8dpi (Fig.15).

Interesantemente, este gene de *AP2* (Medtr2g093060) posee un 80% de similitud con la secuencia del *AP2-1* de frijol el cual es blanco de miR172 y tiene una función importante en el desarrollo del nódulo y la FSN (*Nova-Franco et al., 2015*). Debido a la alta similitud con el *AP2-1* de frijol en este proyecto se denominó *AP2-1* (Medtr2g093060) al de Medicago.

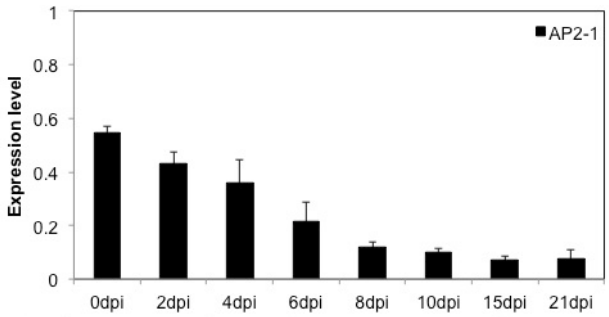


Figura 18. Análisis de la expresión de *AP2-1* en el proceso de nodulación en *Medicago truncatula*. Las plantas de *M. truncatula* fueron inoculadas con *Sinorhizobium meliloti* y colectadas a los días post-inoculación (dpi) señalados. El nivel de expresión se refiere al valor de C_t calculado utilizando al gene EF1 como constitutivo. Los valores representan el promedio ± SD de tres replicas biológicas.

La complementariedad de bases del AP2-1 (Medtr2g093060) con el miR172 de Medicago presenta una calificación de penalidad de 0, es decir que tiene una complementariedad perfecta, lo cual lo hace un buen candidato a ser el gene blanco de miR172.

Una vez identificado a AP2-1 (Medtr2g093060) como el mejor candidato a gene blanco de miR172, se solicitó al servicio de búsqueda de líneas mutantes que posee la Noble Foundation, el cual logró identificar mutantes nulas de este gene mediante la técnica de TAIL PCR. Para ello, se diseñaron oligos específicos de AP2-1 que son utilizados junto con los oligos específicos de Tnt1 para la identificación de mutantes. Así se identificaron dos líneas mutantes de Medicago en el gene *MtAP2-1* (Medtr2g093060) (Tabla 2).

Tabla 2: Lineas mutantes por Tnt1 de *AP2-1* en *Medicago truncatula.*

	Location	Line number
Medtr2g093060	At 115 bp of the exon 1	NF14802
	At 170 bp of the exón 1	NF11780

El gene *AP2-1* de Medicago posee 10 exones y en el último exón se encuentra el sitio de unión a miR172. Su estructura se ejemplifica en la Fig. 19 así como también se ejemplifican en donde se encuentra insertado el transposon Tnt1 en las dos líneas mutantes.

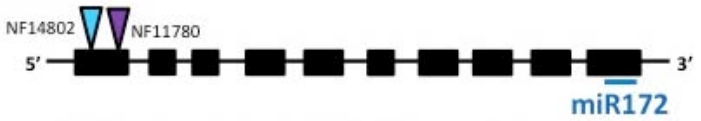


Figura 19. Estructura génica de AP2 de *M. truncatula*. Los cuadros representan los exones mientras que las líneas representan los intrones. Los triángulos representan donde se inserto el transposon en las dos líneas mutantes. Con azul se ejemplifica el sitio de unión para miR172.

Para confirmar el posible efecto positivo en la simbiosis por parte de una mutante nula de *AP2-1* en Medicago. Se analizó el fenotipo simbiótico de las líneas mutantes

obtenidas. Las plantas se crecieron en cuartos de cultivo a temperatura constante 22°C y con un fotoperiodo de 16-8 horas, y se evaluó su fenotipo simbiótico después de 21 días de inoculadas con la cepa *S. meliloti* 1021. Se observó que estas líneas mutantes, generan menos nódulos que las plantas silvestres (≈40 nódulos en la silvestre vs ≈20 nódulos en las líneas mutantes) y la organización de los nódulos en las mutantes se da solo en secciones de la raíz. Además, los nódulos mutantes pierden la forma clásica de un nódulo indeterminado, son redondos y tienen un color blanco lo que indicaría que no están fijando nitrógeno (Fig. 20). El fenotipo de grupos de nódulos contenidos en zonas de la raíces es similar al observado en las mutantes del gene HAR1 en *Lotus japonicus* llamadas "super nodulantes", el ortólogo de NARK involucrado en la AON (Nishimura et al., 2002). Estos resultados son preliminares debido a que se analizaron plantas de T1, que incluye una mezcla de plantas homocigas, heterocigas y silvestres; por lo que experimentos posteriores con generaciones T2 o T3 confirmaran este fenotipo ya que se tendrán solo líneas homocigas.

Figura 20. Fenotipo de nódulos de 21dpi de plantas de *M. truncatula* inoculadas con *S. meliloti*. A, nódulos de plantas silvestres de *M. truncatula*, las flechas rojas indican los nódulos indeterminados, clásicos de Medicago. Ejemplo representativo de nódulos de las dos líneas mutantes NF11780 (B) y NF14802 (C) homocigas.

En base a estos resultados preliminares, se puede concluir que la función del nodo miR172/AP2 en Medicago puede ser diferente a la que posee en frijol y soya, ya que la mutación nula de AP2 no generó un fenotipo simbiótico positivo. Se necesita el análisis simbiótico de las transformantes estables que sobreexpresen a miR172 para poder

confirmar el fenotipo de la mutante nula de AP2 y poder proponer una función para este nodo en el desarrollo del nódulo indeterminado.

CAPITULO VI DISCUSIÓN GENERAL Y PERSPECTIVAS

El nitrógeno es el constituyente mas abundante de un gran numero de compuestos esenciales que intervienen en el funcionamiento de los organismos biológicos. La deficiencia de este elemento en las plantas limita su productividad primaria.

Actualmente este elemento es escaso en los suelos cultivables, a pesar de que constituye el 78% de la atmosfera. Debido a la escasez de este elemento, las practicas agrícolas actuales incluyen el uso de fertilizantes químicos para mantener una alta producción de los cultivos. Esto genera el empobrecimiento de los suelos, junto con la contaminación de agua y aire debido a los residuos químicos de los fertilizantes. Además del alto costo que se tiene al utilizar los fertilizantes químicos. En país desarrollados esto ultimo, puede no ser un problema, pero en países en vías de desarrollo como México, los altos costos del uso de fertilizantes químicos si lo es, ya que los agricultores no pueden costearlo, por lo que las cosechas siguen disminuyendo.

Las leguminosas son consideradas una de las fuentes mas importantes de proteínas de la dieta de consumo tanto animal como humano. Entre estas leguminosas, el frijol, representa el 50% de las leguminosas consumidas a nivel mundial (McClean et al., 2004). En América latina y en África, el frijol, es considerado la primera fuente de proteína de consumo humano (Broughton et al., 2003). Las leguminosas como frijol, poseen la ventaja de formar asociaciones benéficas con las bacterias del suelo, colectivamente denominadas como rhizobias. Las rhizobia forman órganos especializados en las raíces de las leguminosas llamadas nódulos en donde se lleva a cabo la fijación simbiótica de nitrógeno. La fijación simbiótica de nitrógeno es importante desde el punto de vista de la disminución de los fertilizantes químicos y la reducción del costo de producción de cultivos. Además, el uso de cultivos de leguminosas que fijen nitrógeno como el frijol podrían: reducir la contaminación de agua, aumentar la producción de proteína debido a la alta concentración de proteína que poseen las leguminosas, contribuir con nitrógeno

para los siguientes cultivos (rotación de cultivos) e incrementar la fertilidad del suelo (Hardarson 1993).

El frijol como tal, ha sido considerado una leguminosa “pobre” fijadora de nitrógeno comparada con la soya o el haba. A lo largo de los años se han desarrollado varios métodos para medir la fijación de N por parte de los cultivos, como lo son: la actividad de nitrogenasa mediante el método de la reducción de acetileno, el contenido de ureidos y la técnica de la dilución del isotopo ^{15}N. Esta ultima ha sido utilizada ampliamente en diferentes cultivos ya que permite cuantificar, el nitrógeno fijado por parte de la planta y el nitrógeno adquirido del suelo utilizando como referencia una planta crecida en el mismo suelo con fertilizante (De la Mora et al., 2002). Mediante este último método se logró cuantificar la eficiencia de fijación de nitrógeno de diferentes leguminosas y lo que se encontró fue que mientras el haba fija aproximadamente el 70% de nitrógeno y la soya el 60%; frijol solo alcanza a fijar un 30% del nitrógeno atmosférico. (Hardarson 1993). Por esa razón es importante, que el frijol pueda aumentar su capacidad para fijar nitrógeno ya que es un cultivo de importancia en países en vías de desarrollo como México.

Además del problema que representa la deficiencia de nitrógeno en los suelos donde el frijol se cultiva, existen otros problemas a los que se enfrenta en cultivo de frijol, como lo son: enfermedades, insectos, sequía, salinidad. Debido a todo esto, durante muchos años se ha tratado de mejorar a este cultivo por técnicas de fitomejoramiento clásico, lo que ha generado la obtención de cultivos de frijol con una mayor tolerancia a la sequia, resistencia a enfermedades o insectos además de otras características agronómicas importantes (Cipriano et al., 2014). Por otro lado existen varios reportes en donde se ha estudiado el mejoramiento de la fijación de nitrógeno en plantas de frijol. Hardarson (1993) revisa diferentes enfoques para mejorar la fijación de nitrógeno en el cultivo de frijol, desde la inoculación de las semillas previas a ser sembradas con bacterias del genero *Rhizobium* hasta el uso de cepas mejor fijadoras de nitrógeno.

En este trabajo se demostró por primera vez en frijol, que un aumento en la abundancia del miR172c y la consecuente disminución de su gene blanco, el factor de transcripción *AP2-1* provoca un aumento en el número de nódulos de hasta un 50% y un aumento en la fijación de nitrógeno de casi el 60% comparado con las plantas que no sobreexpresaban este miRNA. Recientemente, Yan et al (2013) y Wang et al. (2014) reportaron un efecto similar del miR172 en soya. Por otro lado, se demostró un aumento en la biomasa de las raíces de frijol que sobreexpresan a miR172 incluso en plantas no inoculadas; esto no ha sido reportado para ninguna otra leguminosa incluida soya. Lo anterior podría indicar que, miR172 además de tener un efecto positivo sobre el número de nódulos y fijación de nitrógeno, también lo tiene en el desarrollo de la raíz. El aumento en el crecimiento y el cambio en la arquitectura de la raíz es una respuesta común de las plantas a estrés abiótico como la deficiencia nutricional en el suelo, ya que una mayor área radicular le permite absorber mejor la escasa cantidad de nutrientes presentes.

Además de aumentar el número de nódulos, la fijación de nitrógeno y la biomasa de raíz, en este trabajo se demostró que las plantas de frijol que sobreexpresan a miR172 poseen una tolerancia a nitrato en condiciones de fijación simbiótica de nitrógeno. Esto ha sido una característica deseable por parte de los fitomejoradores de frijol a lo largo de muchos años, debido que se sabe que el nitrato del suelo inhibe la producción de nódulos en las leguminosas (Streeter y Wong 1988). En plantas de frijol que sobreexpresan a miR172, inoculadas con rhizobia en presencia de nitrato (1mM, concentración inhibitoria para plantas de frijol transformadas control) se observó que la fijación de nitrógeno aumenta ligeramente (15%). Esto es importante desde el punto de vista agrícola, ya que adicionando a los suelos cantidades mínimas de fertilizantes nitrogenados (que representa un costo mucho menor), la simbiosis se llevaría a cabo de manera eficiente aumentando el contenido de nitrógeno en la planta.

Todo lo anterior hace del miR172, un regulador importante para ser explorado en estrategias de mejoramiento de la fijación de nitrógeno en frijol para obtener mejor cantidad y calidad del cultivo.

El uso de miR172 para mejor la fijación de nitrógeno en frijol puede discutirse desde dos enfoques principalmente: el enfoque biotecnológico y el genético.

Dentro del enfoque biotecnológico se encuentran varias limitantes como la falta de un procedimiento eficiente para obtener plantas transgénicas estables de frijol. Sin embargo, se han reportado dos técnicas por las cuales el frijol puede ser transformado de manera estable. De hecho, actualmente ya se comercializa en Brasil un frijol transgénico el cual posee una resistencia al virus de mosaico dorado de frijol (Aragao et al., 2013). Este frijol resistente al virus fue generado por bombardeo de partículas, con la cual introdujeron una construcción de RNAi para silenciar genes de involucrados en la replicación viral y en el movimiento sistémico viral. Este mecanismo de utilización de RNAi para inactivar genes blanco de virus es utilizado por el frijol como primer mecanismo de defensa (Aragao et al., 1998). La metodología que se utilizó en este caso, fue el bombardeo de partículas en ejes embrionarios de frijol, los cuales fueron seleccionados con la resistencia al antibiótico neomicina y regenerados in vitro (Aragao et al., 1996).

Por otra parte, Liu et al (2005) reportaron un método de transformación de frijol mediante *Agrobacterium*. Este método introduce a *Agrobaterium tumefaciens* en las hojas de frijol por medio de sonicación e infiltración al vacío. En este reporte, los autores sobreexpresan una proteína LEA (late embryogenesis abundant), la cual le confiere tolerancia al estrés por sequia a las plantas transgénicas de frijol que la poseen.

Utilizando estas técnicas de transformación de frijol, se podrían generar plantas que sobreexpresaran el miR172 o en su defecto que tuvieran silenciado por medio de RNAi el factor de transcripción AP2. Lo cual podría ser evaluado a nivel de campo para

confirmar el fenotipo que se encontró en este trabajo a nivel de invernadero en plantas compuestas de frijol.

El segundo enfoque que puede utilizarse para mejorar el frijol es el genético. El estudio de las mutantes de frijol está aún en una etapa inicial comparado con dichos estudios en las leguminosas modelo Medicago y *L. japonicus*. Recientemente se reportó una población de mutantes de frijol, las cuales fueron generadas por medio de la radiación con “fast-neutron” que provocan deleciones a lo largo del genoma (O’Rourke et al., 2013). Pero aún no esta en proceso la caracterización de plantas mutantes de frijol con características deseables, ya sea en la nodulación u otras. En algún momento a futuro, en estas mutantes se podría buscar y mapear una línea que tuviera una deleción en el gene *AP2-1*, con un fenotipo similar a las sobreexpreantes de miR172, lo cual podrían utilizarse en estudios de campo para evaluar la fijación de nitrógeno.

Además de lo que ya se mencionó acerca de las posibles aplicaciones/ventajas en campo que puede llegar a tener tanto la sobreexpresión de este miRNA como el silenciamiento de su gen blanco *AP2*; también se puede hacer uso de las mutantes de Medicago disponibles para identificar a los genes reguladores rio arriba del nodo miR172-*AP2* y poder entender mas a detalle la función de miR172 en los eventos tempranos de la nodulación.

En Medicago se conoce la vía de señalización de eventos tempranos de nodulación. Esta vía de señalización incluye la percepción de los factores nod por los receptores de NFR1 y NFR5, lo que genera las oscilaciones de calcio que van a permitir la expresión de CCaMK y CYCLOPS para posteriormente activar la expresión de los factores de transcripción NSP1 y NSP2,. Estos activan la expresión de otros factores de transcripción: NIN y ERN1, que a su vez transcriben otros genes necesarios para llevar a cabo la formación del hilo de infección y la consecuente formación del primordio del

nódulo (Oldroyd 2013). Para averiguar cual de estos genes pudieran están regulando la expresión de miR172, el enfoque a seguir seria la evaluación del fenotipo simbiótico, sobreexpresando a miR172 en raíces compuestas de plantas de Medicago que tuvieran una mutación nula en estos genes. Si el fenotipo simbiótico de la sobreexpresión de miR172 en alguna de estas mutantes nulas es diferente al de raíces transgénicas control que sobreexpresan el miR172, quizá similar a las plantas de Medicago silvestres, esto indicaría que el gene mutado estaría regulando la expresión de miR172. También se podría utilizar la mutante en NARK de Medicago para confirmar o no, la hipótesis propuesta para la regulación de miR172 en soya (Wang et al., 2014).

En soya, se reportó la regulación negativa por parte de NARK sobre la expresión de miR172 en la formación del primordio del nódulo. Esto lo demostraron ya que plantas con fenotipo hipernodulante que poseían una mutación en el gene NARK de soya, poseían niveles elevados de miR172 comparadas con las plantas silvestres. Además, esto se confirmó al transformar plantas mutantes de NARK con la sobreexpresión de miR172 lo que provoco un aumento aun mayor del numero de nódulos comparadas con las plantas que solo tenían mutado el gene NARK. Esto confirma que NARK regula negativamente la expresión de miR172 para controlar el numero de nódulos en las raíces de soya (Wang et al., 2014).

En frijol existe una mutante en el gene NARK (Ferguson et al., 2014) fue generada por medio mutagénesis química con Etil Metano Sulfonato (EMS) que posee un fenotipo similar a los de las mutantes supernodulantes de *L. japonicus* o soya (Park y Buttery 1988). Este fenotipo supernodulante se caracteriza por poseer un aumento significativo en el numero de nódulos comparado con plantas silvestres, además de una disminución de la parte aérea, una disminución de la fijación de nitrógeno y de la biomasa de raíz (Bhatia et al., 2001). Esta mutante de frijol podría ayudar a dilucidar el papel de NARK como regulador negativo de miR172 en frijol como ha sido propuesto en soya.

El frijol es un cultivo de gran importancia en México, por lo que la búsqueda de alternativas para su mejoramiento es de suma importancia. El uso de miRNAs para el mejoramiento de leguminosas aun es un campo poco explorado pero podría ser importante debido a que los miRNAs son reguladores globales de diferentes procesos, podrían tener un gran impacto en la tolerancia a diferentes tipos de estrés tanto biótico o biótico así como mejorar el proceso de fijación de nitrógeno en las leguminosas. Las leguminosas transformadas de manera estable con miR172, como frijol, pudieran presentar una mejora en el contenido nutricional de las semillas además de un aumento en los nódulos y fijación de nitrógeno.

Aun existe mucho por explorar acerca de la biología del frijol pero sobre todo hace falta mucho trabajo para desarrollar en México técnicas de transformación para las leguminosas recalcitrantes como lo es frijol; además de obtener nuevas leyes de bioseguridad para poder liberar al campo el frijol transgénico que se pudiera generar como se empezó recientemente a hacer en Brasil.

CAPITULO VII REFERENCIAS

Addo-Quaye C, Eshoo TW, Bartel DP, Axtell MJ (2008) Endogenous siRNA and miRNA targets identified by sequencing of the Arabidopsis degradome. Curr Biol 18: 758-762

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373-399

Aragão FJL, Barros LMG, Brasileiro ACM, Ribeiro SG, Smith FD, Sanford JC, Rech EL (1996) Inheritance of foreign genes in transgenic bean (Phaseolus vulgaris L.) co-transformed via particle bombardment. Theor Appl Genet 93: 142-150

Aragão FJL, Ribeiro SG, Barros LMG, Brasileiro ACM, Maxwell DP, Rech EL, Faria JC (1998) Transgenic beans (Phaseolus vulgaris L.) engineered to express viral antisense RNAs show delayed and attenuated symptoms to bean golden mosaic geminivirus. Mol Breed 4: 491-499

Aragão FJ, Nogueira EO, Tinoco MLP, Faria JC (2013) Molecular characterization of the first commercial transgenic common bean immune to the Bean golden mosaic virus. J Biotechnol 166: 42-50

Arenas-Huertero C, Pérez B, Rabanal F, Blanco-Melo D, De la Rosa C, Estrada-Navarrete G, Sánchez F, Covarrubias AA, Reyes JL (2009) Conserved and novel miRNAs in the legume Phaseolus vulgaris in response to stress. Plant Mol Biol 70**:** 385-401

Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. Plant Cell 15**:** 2730-2741

Axtell MJ, Bartel DP (2005) Antiquity of microRNAs and their targets in land plants. Plant Cell 17: 1658-1673

Bhatia CR, Nichterlein K, Maluszynski M (2001) Mutations affecting nodulation in grain legumes and their potential in sustainable cropping systems. Euphytica 120: 415-432

Bartel DP (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281-297

Benedito VA, Torres‐Jerez I, Murray JD, Andriankaja A, Allen S, Kakar K, Udvardi MK (2008) A gene expression atlas of the model legume Medicago truncatula. Plant J 55: 504-513

Boualem A, Laporte P, Jovanovic M, Laffont C, Plet J, Combier JP, Niebel A, Crespi M, Frugier F (2008) MicroRNA166 controls root and nodule development in Medicago truncatula. Plant J 54**:** 876-887

Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L, Voinnet O (2008) Widespread translational inhibition by plant miRNAs and siRNAs. Science 320: 1185-1190

Broughton WJ, Hernandez G, Blair M, Beebe S, Gepts P, Vanderleyden J (2003) Beans (Phaseolus spp.)–model food legumes. Plant Soil 252: 55-128

Bustos-Sanmamed P, Mao G, Deng Y, Elouet M, Khan GA, Bazin J, Lelandais-Brière, C (2013). Overexpression of miR160 affects root growth and nitrogen-fixing nodule number in Medicago truncatula. Funct Plant Biol 40**:** 1208-1220.

Cartolano M, Castillo R, Efremova N, Kuckenberg M, Zethof J, Gerats T, Vandenbussche M (2007) A conserved microRNA module exerts homeotic control over Petunia hybrida and Antirrhinum majus floral organ identity. Nat Genet 39: 901-905

Chen X (2004) A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science 303**:** 2022–2025

Chen X (2009) Small RNAs and their roles in plant development. Annu Rev Cell Dev 25: 21-44.

Combier JP, Frugier F, de Billy F, Boualem A, El-Yahyaoui F, Moreau S, Vernié T, Ott T, Gamas P, Crespi M, Niebel A (2006) MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in Medicago truncatula. Genes Dev 20**:** 3084-3088

Cosson V, Durand P, d'Erfurth I, Kondorosi A, Ratet P (2006) Medicago truncatula transformation using leaf explants. In Agrobacterium Protocols 115-128

Cipriano TM, Ibrahim AB, Faria JC, FJL Aragao (2014) Development of transgenic common bean with agronomic traits. Journal Integrative Legume Society 2: 27-29

Erfurth I, Cosson V, Eschstruth A, Lucas H, Kondorosi A, Ratet P (2003) Efficient transposition of the Tnt1 tobacco retrotransposon in the model legume Medicago truncatula. Plant J 34: 95-106

D'haeseleer K, Den Herder G, Laffont C, Plet J, Mortier V, Lelandais‐Brière C, De Bodt S, De Keyser A, Crespi M, Holsters M, Frugier F, Goormachtig S (2011) Transcriptional and post‐transcriptional regulation of a NAC1 transcription factor in Medicago truncatula roots. New Phytol 191**:** 647-661

Dai X, Zhao PX (2011) psRNATarget: a plant small RNA target analysis server. Nucleic Acids Res 39**:** W155-W159

De la Mora BV, Cadisch G, Schunemann AA (2002). Comparison of isotope methodologies to assess N_2 fixation and its fate in plants and soils. Agrociencia 37: 117-128

De Luis A, Markmann K, Cognat V, Holt DB, Charpentier M, Parniske M, Stougaard J, Voinnet O (2012) Two microRNAs linked to nodule infection and nitrogen-fixing ability in the legume Lotus japonicus. Plant Physiol 160**:** 2137-2154

Dong Z, Shi L, Wang Y, Chen L, Cai Z, Wang Y, Jin J, Li X (2013) Identification and dynamic regulation of microRNAs involved in salt stress responses in functional soybean nodules by high-throughput sequencing. IJMS 14**:** 2717-2738

Doss RP (2005) Treatment of pea pods with Bruchin B results in up-regulation of a gene similar to MtN19. Plant Physiol Biochem 43: 225-23

Ebhardt HA, Fedynak A, Fahlman RP (2010) Naturally occurring variations in sequence length creates microRNA isoforms that differ in argonaute effector complex specificity. Silence 1:12

Ferguson BJ, Indrasumunar A, Hayashi S, Lin HM, Lin, YH, Reid DE, Gresshoff PM (2010) Molecular analysis of legume nodule development and autoregulation. J Integr Plant Biol 52**:** 61-76

Ferguson BJ, Li D, Hastwell AH, Reid DE, Li Y, Jackson SA, Gresshoff PM (2014) The soybean (Glycine max) nodulation-suppressive CLE peptide, GmRIC1, functions interspecifically in common white bean (Phaseolus vulgaris), but not in a supernodulating line mutated in the receptor PvNARK. Plant Biotechnol J 12: 1085-1097

Formey D, Sallet E, Lelandais-Brière C, Ben C, Bustos-Sanmamed P, Niebel A, Frugier F, Combier JP, Debelle F, Hartmann C, Poulain J, Gavory F, Wincker P, Rpux C, Gentzbittel L, Gouzy J, Crespi, M (2014) The small RNA diversity from Medicago truncatula roots under biotic interactions evidences the environmental plasticity of the miRNAome. Genome Biol 15: 457

Formey D, Iñiguez L, Peláez P, Li YF, Sunkar R, Sánchez F, Reyes JL, Hernández G (2015) Genome-wide identification of the Phaseolus vulgaris sRNAome using small RNA and degradome sequencing. BMC Genomics. Enviado

Franco AA, Munns DN (1982) Nodulation and growth of Phaseolus vulgaris in solution culture. Plant Soil 66**:** 149-160

Franco-Zorrilla JM, Valli A, Todesco M, Mateos I, Puga MI, Rubio-Samoza I, Leyva A, Weigel D, García JA, Paz-Ares J (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. Nature Genetics 39: 1033-1037

Gamas P, de Carvalho Niebel F, Lescure N, Cullimore JV (1996) Use of a subtractive hybridization approach to identify new Medicago truncatula genes induced during root nodule development. Molec Plant-Microbe Interact 9: 233-242

Girard L, Valderrama B, Palacios R, Romero D, Dávila G (1996) Transcriptional activity of the symbiotic plasmid of Rhizobium etli is affected by different environmental conditions. Microbiol 142**:** 2847-2856

Girard L, Brom S, Dávalos A, López O, Soberón M, Romero D (2000) Differential regulation of fixN-reiterated genes in Rhizobium etli by a novel fixL-fixK cascade. Molec Plant-Microbe Iinteract 13: 1283-1292

Graham PH, Vance CP (2003) Legumes: importance and constraints to greater use. Plant Physiol 131: 872-877

Hardarson G (1993) Methods for enhancing symbiotic nitrogen fixation. In Enhancement of Biological Nitrogen Fixation of Common Bean in Latin America 1-17 Springer Netherlands

Hoffmann B, Trinh TH, Leung J, Kondorosi A, Kondorosi E (1997) A new Medicago truncatula line with superior in vitro regeneration, transformation, and symbiotic properties isolated through cell culture selection. Molec Plant-Microbe Interact 10: 307-315

Jagadeeswaran G, Zheng Y, Li YF, Shukla LI, Matts J, Hoyt P, Sunkar R (2009) Cloning and characterization of small RNAs from Medicago truncatula reveals four novel legume-specific microRNA families. New Phytol 184: 85-98

Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol cell 14**:** 787-799

Jones-Rhoades MW (2012) Conservation and divergence in plant microRNAs. Plant Mol Biol 80: 3-16

Jung JH, Seo YH, Seo PJ, Reyes JL, Yun J, Chua NH, Park CM (2007) The GIGANTEA-regulated microRNA172 mediates photoperiodic flowering independent of CONSTANS in Arabidopsis. Plant Cell 19: 2736-2748

Kidner CA, Martienssen RA (2005) Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. Nature 428: 81-84

Kim VN (2005) Small RNAs: classification, biogenesis, and function. Mol Cells 19: 1-15

Kimura M, Yamamoto YY, Seki M, Sakurai T, Sato M, Abe T, Matsui M (2003) Identification of Arabidopsis Genes Regulated by High Light–Stress Using cDNA Microarray. Photochem Photobiol 77: 226-233

Lauter N, Kampani A, Carlson S, Goebel M, Moose SP (2005) microRNA172 down-regulates glossy15 to promote vegetative phase change in maize. Proc Nalt Acad Sci 102: 9412-9417

Lee H, Yoo SJ, Lee JH, Kim W, Yoo SK, Fitzgerald H, Ahn JH (2010) Genetic framework for flowering-time regulation by ambient temperature-responsive miRNAs in Arabidopsis. Nucleic Acids Res, gkp1240

Lelandais-Briére C, Naya L, Sallet E, Calenge F, Frugier F, Hartmann C, Gouzy J, Crespi M (2009) Genome-wide Medicago truncatula small RNA analysis revealed novel microRNAs and isoforms differentially regulated in roots and nodules. Plant Cell 21**:** 2780–2796

Li H, Deng Y, Wu T, Subramanian S, Yu O (2010) Misexpression of miR482, miR1512, and miR1515 increases soybean nodulation. Plant Physiol 153**:** 1759-1770

Liu Z, Park BJ, Kanno A, Kameya T (2005) The novel use of a combination of sonication and vacuum infiltration in Agrobacterium-mediated transformation of kidney bean (Phaseolus vulgaris L.) with lea gene. Mol Breed 16: 189-197

Magori S, Kawaguchi M (2009) Long-distance control of nodulation: molecules and models. Mol Cells 27**:** 129-134

Marchal K, Vanderleyden, J (2000) The" oxygen paradox" of dinitrogen-fixing bacteria. Biol Fert Soils 30: 363-373

McClean P, Kami J, Gepts P (2004) Genomics and genetic diversity in common bean. Legume Crop Genomics 60-82

Miransari M (2014) The interactions of soil microbes, arbuscular mycorrhizal fungi and N-fixing bacteria, Rhizobium, under different conditions including stress. In Use of Microbes for the Alleviation of Soil Stresses Pag: 1-21

Murray JD (2011) Invasion by invitation: Rhizobial infection in legumes. Mol Plant-Microbe Interact 24**:** 631-639

Naya L, Paul S, Valdés-López O, Mendoza-Soto AB, Nova-Franco B, Sosa-Valencia G, Reyes JL, Hernández G (2014) Regulation of Copper Homeostasis and Biotic Interactions by MicroRNA 398b in Common Bean. PloS One 9: e84416

Nishimura R, Hayashi M, Wu GJ, Kouchi H, Imaizumi-Anraku H, Murakami Y, Kawaguchi M (2002) HAR1 mediates systemic regulation of symbiotic organ development. Nature 420: 426-429

Nova-Franco B, Íñiguez LP, Valdés-López O, Alvarado-Affantranger X, Leija A, Fuentes S. I, Ramírez M, Paul S, Reyes JL, Girard L, Hernández, G (2015) The miR172c-AP2-1 Node as a Key Regulator of the Common Bean-Rhizobia Nitrogen Fixation Symbiosis Plant Physiol pp-114

Oldroyd GED, Downie JA (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol 59**:** 519-546

Oldroyd GE, Murray JD, Poole PS, Downie JA (2011) The rules of engagement in the legume-rhizobial symbiosis. Annu Rev Genet 45: 119-144

Oldroyd GED (2013) Speak, friend and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbiol 11**:** 252-263

Okamoto S, Ohnishi E, Sato S, Takahashi K, Nakazono M, Tabata S, Kawaguchi M (2009) Nod factor/nitrate-induced CLE genes that drive HAR1-mediated systemic regulation of nodulation. Plant Cell Physiol 50**:** 67-77

O'Rourke JA, Iniguez LP, Bucciarelli B, Roessler J, Schmutz J, McClean PE, Jackson SA, Hernandez G, Graham MA, Stupar RM, Vance CP (2013). A re-sequencing based assessment of genomic heterogeneity and fast neutron-induced deletions in a common bean cultivar. Front Plant Sci 4

Paredes-López O, Lara FG, Pérez LAB (2006) Los alimentos mágicos de las culturas indígenas mesoamericanas. Fondo de Cultura Económica Vol. 212

SJ, Buttery BR (1988) Nodulation mutants of white bean (Phaseolus vulgaris L.) induced by ethyl-methane sulphonate. Can J Plant Sci 68: 199-202

Park W, Li J, Song R, Messing J, Chen X (2002) CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. Curr Biol 12: 1484-1495

Paul S, Kundu A, Pal A (2011) Identification and validation of conserved microRNAs along with their differential expression in roots of Vigna unguiculata grown under salt stress. PCTOC 105: 233-242

Peláez P, Trejo MS, Iñiguez LP, Estrada-Navarrete G, Covarrubias AA, Reyes JL, Sánchez F (2012) Identification and characterization of microRNAs in Phaseolus vulgaris by high-throughput sequencing. BMC Genomics 13**:** 83

Ramachandran V, Che X. (2008) Degradation of microRNAs by a family of exoribonucleases in Arabidopsis. Science 321: 1490-1492.

Ramírez M, Flores-Pacheco G, Reyes JL, Alvarez AL, Drevon JJ, Girard L, Hernández G (2013) Two common bean genotypes with contrasting response to phosphorus deficiency show variations in the microRNA 399-mediated PvPHO2 regulation within the PvPHR1 signaling pathway. IJMS 14: 8328-8344

Reid DE, Ferguson BJ, Hayashi S, Lin Y-H, Gresshoff PM (2011) Molecular mechanism controlling legume autoregulation of nodulation. Ann Bot 108**:** 789-795

Reid DE, Li D, Ferguson BJ, Gresshoff PM (2013) Structure–function analysis of the GmRIC1 signal peptide and CLE domain required for nodulation control in soybean. J Exp Bot ert008

Rogers K, Chen X (2013) Biogenesis, turnover, and mode of action of plant microRNAs. Plant Cell 25: 2383-2399

Roponen I (1970) The effect of darkness on the leghemoglobin content and amino acid levels in the root nodules of pea plants. Physiol Plant 23: 452-460

Rubio-Somoza I, Weigel D (2011) MicroRNA networks and developmental plasticity in plants. Trends Plant Sci 16: 258-264

Schmutz J, McClean PE, Mamidi S, Wu GA, Cannon SB, Grimwood J, Jenkins J, Shu S, Song Q, Chavarro C, Torres-Torres M, Geffroy V, Moghaddam SM, Gao D, Abernathy B, Barry K, Blair M, Brick MA, Chovatia M, Gepts P, Goodstein DM, Gonzales M, Hellsten U, Hyten DL, Jia G, Kelly JD, Kudrna D, Lee R, Richard MMS, Miklas PN, Osomo JM, Rodrigues J, Thareau V, Urrea CA, Wang M, Yu Y, Zhang M, Wing RA, Cregan PB, Rokhsar DS, Jackson SA (2014) A reference genome for common bean and genome-wide analysis of dual domestication. Nat Genet PMID: 24908249

Schmid M, Uhlenhaut NH, Godard F, Demar M, Bressan R, Weigel D, Lohmann JU (2003) Dissection of floral induction pathways using global expression analysis. Development 130**:** 6001-6012

Shamseldin A (2013) The role of different genes involved in symbiotic nitrogen fixation-Review. GJBB 8: 84-94

Smith PM, Atkins CA (2002) Purine biosynthesis. Big in cell division, even bigger in nitrogen assimilation. Plant Physiol 128: 793-802

Song QX, Liu YF, Hu XY, Zhang WK, Ma B, Chen SY, Zhang JS (2011) Identification of miRNAs and their target genes in developing soybean seeds by deep sequencing. BMC Plant Biol 11**:** 5

Soyano T, Hirakawa H, Sato S, Hayashi M, Kawaguchi M (2014) NODULE INCEPTION creates a long-distance negative feedback loop involved in homeostatic regulation of nodule organ production. Proc Natl Acad Sci, 111: 14607-14612

Streeter J, Wong PP (1988) Inhibition of legume nodule formation and N_2 fixation by nitrate. Crit Rev Plant Sci 7**:** 1-23

Sunkar R, Zhou X, Zheng Y, Zhang W, Zhu JK (2008) Identification of novel and candidate miRNAs in rice by high throughput sequencing. BMC Plant Biol 8: 25

Sunkar R, Kapoor A, Zhu JK (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell 18: 2051-2065

Subramanian S, Fu Y, Sunkar R, Barbazuk WB, Zhu JK, Yu O (2008) Novel and nodulation-regulated microRNAs in soybean roots. BMC Genomics 9**:** 160

Timmers AC, Soupène E, Auriac MC, de Billy F, Vasse J, Boistard P, Truchet G (2000) Saprophytic intracellular rhizobia in alfalfa nodules. Mol Plant Microbe Interact 13: 1204-1213

Town CD (2006) Annotating the genome of Medicago truncatula. Curr Opin Plant Biol 9: 122-127

Turner M, Yu O, Subramanian S (2012) Genome organization and characteristics of soybean microRNAs. BMC Genomics 13:169

Turner M, Nizampatnam NR, Baron M, Coppin S, Damodaran S, Adhikari S, Arunachalam SP, Yu O, Subramanian S (2013) Ectopic expression of miR160 results in auxin hypersensitivity, cytokinin hyposensitivity, and inhibition of symbiotic nodule development in soybean. Plant Physiol 162**:** 2042-2055.

Udvardi M, Poole PS (2013) Transport and metabolism in legume-rhizobia symbioses. Annu Rev Plant Biol 64: 781-805

Van de Velde W, Guerra JCP, De Keyser A, De Rycke R, Rombauts S, Maunoury N, Goormachtig S (2006) Aging in legume symbiosis. A molecular view on nodule senescence in Medicago truncatula. Plant Physiol 141: 711-720

Valdés-López O, Yang SS, Aparicio-Fabre R, Graham PH, Reyes JL, Vance CP, Hernández G (2010) MicroRNA expression profile in common vean (Phaseolus vulgaris) under nutrient deficiency stresses and manganese toxicity. New Phytol 187**:** 805–818

Valdés-López O, Arenas-Huertero C, Ramírez M, Girard L, Sánchez F, Vance CP, Reyes JL, Hernández G (2008) Essential role of MYB transcription factor: PvPHR1 and microRNA: PvmiR399 in phosphorus deficiency signaling in common bean roots. Plant Cell Environ 31**:** 1834–1843

Vázquez F (2009) Small RNAs in Plants eLS.

Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. Cell 136: 669-687

Wang Y, Li P, Cao X, Wang X, Zhang A, Li X (2009) Identification and expression analysis of miRNAs from nitrogen-fixing soybean nodules. Biochem Biophys Res Commun 378**:** 799-803

Wang Y, Wang L, Zou Y, Chen L, Cai Z, Zhang S, Zhao F, Tian Y, Jiang Q, Ferguson BJ, Gresshoff PM, Li X (2014) Soybean miR172c targets the repressive AP2 transcription factor NNC1 to activate ENOD40 expression and regulate nodule initiation. Plant Cell 26: 4782-4801

Wu G, Park MY, Conway SR, Wang JW, Weigel D, Poethig RS (2009) The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell 138: 750-759

Yamasaki H, Abdel-Ghany SE, Cohu CM, Kobayashi Y, Shikanai T, Pilon M (2007). Regulation of copper homeostasis by micro-RNA in Arabidopsis. J Biol Chem 282: 16369-16378

Yan Z, Hossain MS, Wang J, Valdés-López O, Liang Y, Libault M, Lijuan Q, Stacey G (2013) miR172 regulates soybean nodulation. Mol Plant-Microbe Interact 26**:** 1371-1377

Yoshikawa M, Peragine A, Park MY, Poethig RS (2005) A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. Genes & development 19: 2164-2175

Yoshikawa T, Ozawa S, Sentoku N, Itoh JI, Nagato Y, Yokoi S (2013) Change of shoot architecture during juvenile-to-adult phase transition in soybean. Planta 238**:** 229-237

Yu B, Bi L, Zheng B, Ji L, Chevalier D, Agarwal M, Ramachandran V, Li W, Lagrange T, Walker JC, Chen, X. 2008. The FHA domain proteins DAWDLE in Arabidopsis and SNIP1 in humans act in small RNA biogenesis. Proc Natl Acad Sci 105: 10073–10078

Zhu QH, Spriggs A, Matthew L, Fan L, Kennedy G, Gubler F, Helliwell C (2008) A diverse set of microRNAs and microRNA-like small RNAs in developing rice grains. Genome Res 18: 1456-1465

Zhu QH, Upadhyaya NM, Gubler F, Helliwell CA (2009) Over-expression of miR172 causes loss of spikelet determinacy and floral organ abnormalities in rice (Oryza sativa). BMC Plant Biol 9: 149

Zhu QH, Helliwell CA (2010) Regulation of flowering time and floral patterning by miR172. J Exp Bot 62**:** 487-495

CAPITULO VIII: ANEXOS

VIII. 1 ANEXO I

Hernández G & Nova-Franco B. (2014) MicroRNAs as post-transcriptional regulators in common bean (*Phaseolus vulgaris*). *The journal of the International Legume Society.* 2: 30-32

LEGUME SOCIETY

LEGUME PERSPECTIVES

Phaseolus: A New World gift to mankind

Why common beans are so common?

The journal of the International Legume Society

Issue 2 • April 2014

IMPRESSUM

ISSN
2340-1559

Quarterly publication
January, April, July and October,
with an open possibility of additional issues

Published by
International Legume Society

Office
CSIC, Institute for Sustainable Agriculture
Apdo. 4084, 14080 Córdoba, Spain
Phone: +34957499215 • Fax: +34957499252

Subscriptions
Office
(diego.rubiales@ias.csic.es)

Cover photo
Marta Santalla Ferradas

FOR AUTHORS

Legume Perspectives is an international peer-reviewed journal aiming to interest and inform a worldwide multidisciplinary readership on very different aspects of legume research and use, including genetics, agronomy, animal production, human nutrition and health and economics.

Please write your article so that it will assist in the exchange of information between people working in different expert fields of legume research: write to the length requested; provide a review of the most important information on your topic; try to avoid (or explain) specialist words, unusual jargon and acronyms; emphasise results and conclusions; choose titles carefully and add subheadings that tell readers something. *Legume Perspectives* prefers a clear, simple and comprehensive writing style that would make its articles interesting and useful for both academic and amateur audience. Your manuscripts does not have to follow the usually strict structure of the research papers.

We strongly encourage you to submit either longer (1,250 words + 2 tables, figures or photos + up to 8 references) or shorter (450 words + 1 table, figure, photograph or drawing + up to 4 references) manuscripts. The Editorial Board may allow any variation from case to case. Please provide also an additional abstract of between 90 and 100 words and from 4 to 6 key words listed alphabetically.

The manuscripts should be prepared in Microsoft Office Word, using Times New Roman font, 12 points size. The references should be listed and numbered alphabetically, following the style of any of the published papers in this issue. The tables may be incorporated in the manuscript, while it is desirable that figures, photographs or drawings are submitted separately preferably as jpg files.

Please send your prepared manuscripts or any inquiries on publishing in *Legume Perspectives* to diego.rubiales@ias.csic.es or legume.society@gmail.com

EDITORIAL

I am proud to present this Legume Perspectives *issue dedicated to Common Bean, one of the most important grain legume crops for direct human consumption in the world. The origin and diversity of the crop is examined along with the current state of genetic information and the status of breeding programs worldwide. Articles describing beneficial associations with soilborne microbes leading to an increased productivity, biotic and abiotic stresses that affect this crop and breeding for beneficial traits to improve human health are also included. Hopefully, this collection of interesting articles will encourage and stimulate not only those knowledgeable readers, but to those who would like to become familiarized with this fascinating legume. I would like to take the opportunity to thank all colleagues who have made this issue possible: the authors of the articles, and the associated Phaseomics research teams, for their insightful contributions, and sincere acknowledgment to those people who contributed to produce this issue.*

Marta Santalla
Managing Editor of
Legume Perspectives *issue 2*

CONTENTS

MicroRNAs as post-transcriptional regulators in common bean (*Phaseolus vulgaris*)

by Georgina HERNÁNDEZ* and Bárbara NOVA-FRANCO

Abstract: Current knowledge on plant microRNAs (miRNAs) comes mostly from *Arabidopsis*. The majority of plant miRNAs targets are transcription factors. MiRNAs have been involved as relevant global regulator of plant developmental processes as well as response / adaptation to different types of biotic and abiotic stresses. Despite the agronomic importance of legume plants, the knowledge of roles of miRNAs in legumes, including common bean (*Phaseolus vulgaris*), is scant. MiRNAs are involved in biological processes like nutrient balance, development, reproduction and plant-microbe interactions; therefore we consider that research in *P. vulgaris* miRNAs is crucial for improvement of this staple crop. Here we will review recent information about miRNAs in common bean that has been derived from research groups of the National University of Mexico.

Key words: common bean, microRNAS, nitrogen fixation, abiotic stress, metal toxicty.

MiRNA biogenesis in plants

Complex biological processes such as plant development or plant adaptation to variable environmental conditions are finely and precisely controlled by multiple regulatory mechanisms. These include transcriptional and post-transcriptional regulation of gene expression where transcription factors and non-protein-coding RNAs play key roles.

The microRNAs (miRNAs) are small non-protein-coding RNAs that have emerged as ubiquitous post-transcriptional gene regulatory molecules in plants and animals. Plant miRNAs, approximately 21 nucleotides long, are derived from the processing of longer primary miRNA transcripts adopting hairpin-like structures. MiRNAs are negative regulators that suppress expression of their target mRNA mainly by inducing its degradation. The recognition of the miRNA target(s) is based in sequence complementarity (3) (Fig. 1).

Phaseolus vulgaris miRNAs

The first studies to identify miRNAs included the cloning and sequencing - employing traditional sequencing methods- of populations of small RNAs present in different plants. This strategy was used to identify miRNAs from common bean, both from different organs of plants grown in optimal conditions and from seedlings subjected to abiotic stresses such as drought, cold and salinity (1). Members from 16 conserved miRNAs families and eight novel miRNAs were identified in common bean. More recently, deep-sequencing technologies have allowed identifying larger numbers of plant miRNAs. High-throughput small RNA sequencing was applied to extend our knowledge of the common bean miRNA population (6). Small RNA libraries were prepared from common bean roots, seedling, flower buds, and leaves and these were sequenced using Illumina's platform. In this work 109 miRNAs belonging to 29 conserved families were identified for *P. vulgaris* and 29 novel miRNA candidates were predicted based on small RNA reads and precursor predictions (6). Bioinformatic analyses have been used to predict target genes for conserved and novel common bean RNAs (1, 6). As shown in Table 1, most of the predicted targets for conserved miRNAs that have been detected in *P. vulgaris* code for transcritpion factors belonging to different gene families. However other miRNAs recognize targets with variable functions *i.e.* pvu-miR2119 targets an alcohol dehydrogenase mRNA.

Universidad Nacional Autónoma de México, Centro de Ciencias Genómicas, Cuernavaca, Mexico (gina@ccg.unam.mx)

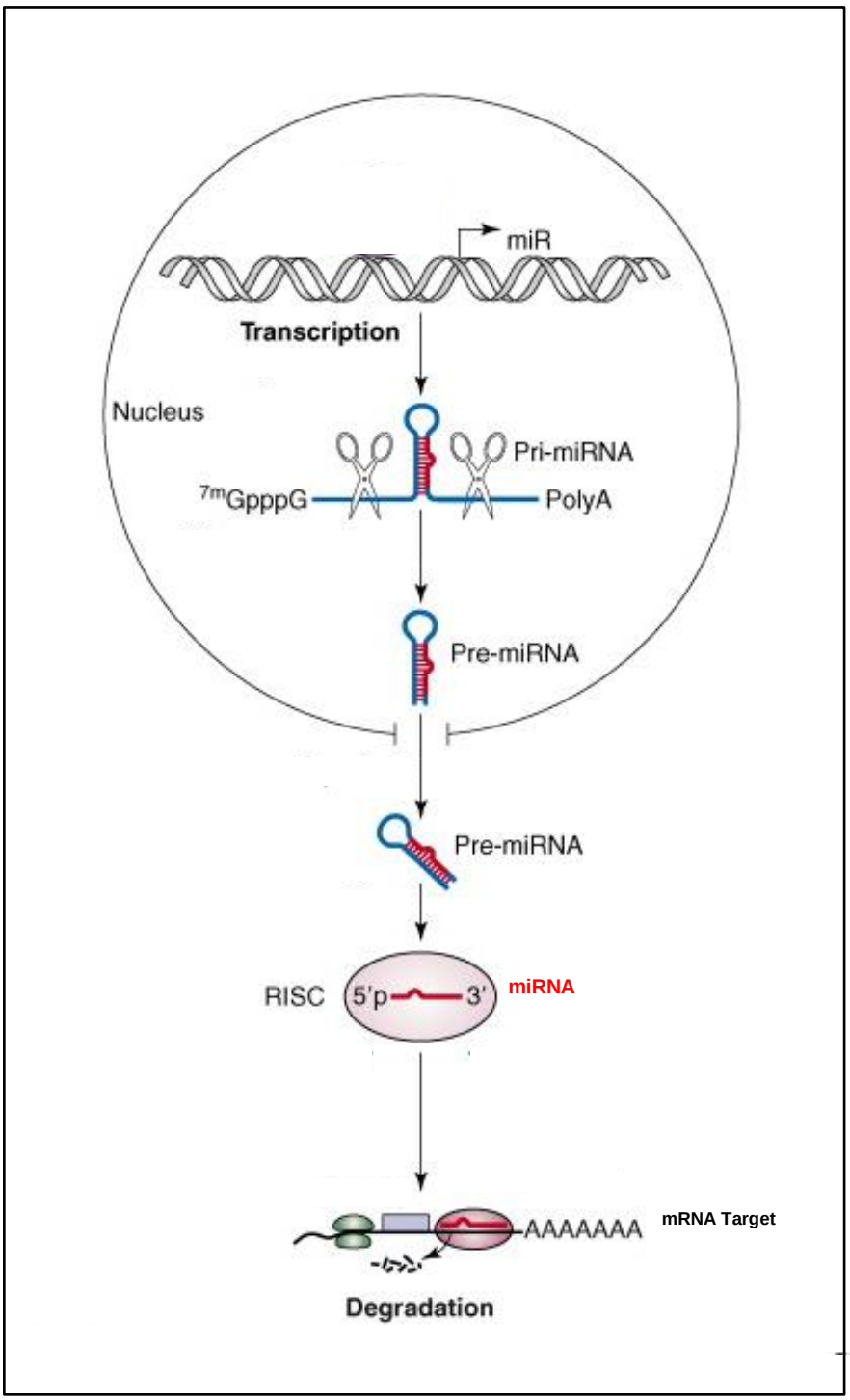


Figure 1. MiRNA biogenesis in plants. MIR genes are transcribed from their own locus. Precursors are processed to produce the mature miRNA that incorporates into RISC (RNA induced silencing complex), binds specifically to a target mRNA and induces its degradation. Modified from Zhao et al. (2007) Trends Biochem Sci 32

Published work about identification of miRNAs in common bean (1, 6) was done prior to having access to the genome sequence of this legume. However *P. vulgaris* genome was sequenced recently and it is now deposited in Phytozome database (*Phaseolus vulgaris* v0.9, DOE-JGI and USDA-NIFA, http::/www.phytozome.net/commonbean). This resource allows mapping the miRNA-coding genes in the *Phaseolus* genome and identifying the miRNAs precursors with predicted stem-loop structure.

Research in our group aims to define the roles of miRNAs in common bean nodule development/function and abiotic stress responses. The root nodules are novel plant organs that result from effective interactions between rhizobia, nitrogen (N)-fixing bacteria, and legumes. The differentiated N-fixing bacteroids establish in the nodules and fix atmospheric N that is assimilated, as ammonia, by the plant. Through this symbiotic interaction legumes produce their own fertilizer, and so it is relevant for sustainable agriculture. Common bean symbiotic N-fixation and crop yield is limited by abiotic stresses such as nutrient deficiency and metal toxicity that are common in acidic soils where beans are grown. Current knowledge indicates that legume miRNAs play important regulatory roles in processes like rhizobia-interaction, N-fixation and nodule development as well as in the mechanisms that allow common bean plants to cope with environmental stresses (7). We consider that the understanding of miRNAs roles in regulatory networks is relevant for common bean improvement.

We have analyzed the expression profile of miRNAs in leaves roots and nodules of nutrient-sufficient and nutrient-stressed (phosphorus, iron or nitrogen deficiency; and (aluminum, manganese or copper toxicity) common bean plants. For this we used a hybridization approach employing miRNA macroarrays printed with oligonucleotides complementary to known miRNAs from *P. vulgaris*, *Glycine max* (soybean) and miRNAs conserved in different plants (9). We have detected 42 miRNAs expressed in the different common bean organs and stress conditions. Recently we have identified the genes coding for these miRNAs in the *P. vulgaris* genome. Some miRNAs responded to nearly all stresses and in the three organs analyzed while others showed organ specific responses.

As mentioned before, our group is most interested in identifying relevant regulatory roles of miRNAs in the N-fixing nodules of common bean. Table 1 shows 19 miRNAs identified as nodule-expressed in *P. vulgaris,* via miRNA macroarray hybridization (9). These include conserved and *P.vulgaris* miRNAs. Analysis of the *P. vulgaris* genome sequence (http::/www.phytozome.net/commonbean) lead us to identify the number of loci that code for each of this miRNAs, which vary from 1 to 9, thus indicating the existence of different members of each miRNA family. Most of these miRNAs were also expressed in roots and/or in leaves, with the exception of miR172 that was detected only in the nodules. Two conserved miRNAs: miR319 and miR398 were detected only in stressed nodules (nutrient deficiency or metal toxicity) and not in nodules from plants in optimal growth conditions, thus indicating their role in stress responses.

We have demonstrated the participation of PvmiR399 in the PvPHR1 transcription factor signaling pathway for phosphorus (P)-deficiency in common bean (8). For this we used a functional genomics approach applying the RNAi technology in bean composite plants, with untransformed shoots and transformed roots resulting from *Agrobacterium rhizogenes* - mediated transformation (2, 8). We analyzed the transcript profile of genes that respond to P-deficiency in composite plants with low (silenced) transcript levels of PvPHR1 and PvmiR399 growing in P-deficiency as compared to optimal conditions. Our results demonstrated that, similar than in *Arabidopsis,* PvPHR1 controls P-deficiency signaling in common bean roots. Once P-deficiency is sensed -either locally or systemically- by unknown molecule(s) the PvPHR1 transcription factor positively regulates the expression of target P-responsive genes (for P transport, remobilization and homeostasis) and also of PvmiR399. The target of PvmiR399 is the ubiquitin E3 conjugase PvPHO2 that promotes degradation of some P-deficiency responsive genes through ubiquitination. In P-deficient conditions PvmiR399 will increase and so will exert a negative regulation upon PvPHO2 to prevent degradation of genes needed to cope with P-stress.

Table 1. MiRNAs in *Phaseolus vulgaris* nodules

miRNA	Target gene	miR loci in *P. vulgaris* genome [a]	miRNA expression in *P. vulgaris* [b]
miR156	Squamosa promotor binding-like protein (SPL)	5	N, R, L
miR157	Squamosa promotor binding-like protein (SPL)	6	N, R, L
miR159	MYB transcription factors	1	N, R, L
miR160	Auxin Response Factors (ARFs)	5	N, R, L
miR164	NAC, CUP Transcription factors	7	N, R, L
miR166	ATHBs	9	N, R, L
miR167	Auxin Response Factors (ARFs)	5	N, L
miR170	Scarecrow-like protein	6	N, R, L
miR172	APETALA 2 (AP2)	6	N
miR319	TCP Transcription factors	2	SN, L
miR390	Trans-Acting siRNA 3 (TAS3)	2	N, R, L
miR395	ATP Sulfurylase	4	N, R, L
miR396	GRL	2	N, R, L
miR398	Cu Superoxide Dismutase	2	SN, R, L
pvu-miR159.2	Chlatrin heavy chain	1	N, R, L
pvu-miR1511	SPIRAL1-like1	0	N, L
pvu-miR2118	U1 snRNP 70K	1	N, R, L
pvu-miR2119	Alcohol dehydrogenase	1	N, R, L
pvu-miR2199	ARF-GAP	1	N, R, L

Current research from our group aims to demonstrate the role of selected miRNAs in the nodulation and symbiotic process of common bean and also in the response of the plant to metal toxicity stress (5). There is still a lot to know about the crucial roles of miRNAs in common bean. We are confident that knowledge in this area will expand in the near future and it will contribute to improve yield and quality of the most important grain legume for human consumption in the world. ■

References

(1) Arenas-Huertero C, Pérez B, Rabanal F, Blanco-Melo D, De la Rosa C, Estrada-Navarrete G, Sánchez F, Covarrubias AA, Reyes JL (2009) Conserved and novel miRNAs in the legume *Phaseolus vulgaris* in response to stress. Plant Mol Biol 70:385-401

(2) Estrada-Navarrete G, Alvarado-Affantranger X, Olivares J-E, Guillén G, Díaz-Camino C, Campos F, Quinto C, Gresshoff PM, Sanchez F (2007) Fast, efficient and reproducible genetic transformation of *Phaseolus* spp. by *Agrobacterium rhizogenes*. Nat Protoc 2:1819-1824

(3) Jones-Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAS and their regulatory roles in plants. Ann Rev Plant Biol 57:19-53

(4) Mallory AC, Vaucheret H (2006) Functions of microRNAs and related small RNAs in plants. Nat Genet 38:S31-S36

(5) Mendoza-Soto AB, Sánchez F, Hernández G (2012) MicroRNAs as regulators in plant metal toxicity response. Front Plant Sci 3:105

(6) Peláez P, Trejo MS, Íñiguez LP, Estrada-Navarrete G, Covarrubias AA, Reyes JL, Sánchez F (2012) Identification and characterization of microRNAs in *Phaseolus vulagris* by high-throughput sequencing. BMC Genomics 13:83

(7) Simon SA, Meyers BC, Sherrier DJ (2009) MicroRNAs in the rhizobia legume symbiosis. Plant Physiol 151:1002-1008

(8) Valdés-López O, Arenas-Huertero C, Ramírez M, Girard L, Sánchez F, Vance CP, Reyes JL, Hernández G (2008) Essential role of MYB transcription factor: PvPHR1 and microRNA: PvmiR399 in phosphorus-deficiency signaling in common bean roots. Plant Cell Environ 31:1834-1843

(9) Valdés-López O, Yang SS, Aparicio-Fabre R, Graham, PH, Reyes JL, Vance CP, Hernández G (2010) MicroRNA expression profile in common bean (*Phaseolus vulgaris*) under nutrient deficiency stresses and manganese toxicity. New Phytol 187:805–818

(10) Zhao Y, Srivastava D (2007) A developmental view of microRNA function. Trends Biochem Sci 32:189-197

VIII.2 ANEXO II

Naya L, Paul S, Valdés-López O, Mendoza-Soto AB., Nova-Franco B, Sosa-Valencia G, Reyes JL, Hernández G. (2014). Regulation of Copper Homeostasis and Biotic Interactions by MicroRNA 398b in Common Bean. *PloS one*, *9*(1), e84416.

OPEN ACCESS Freely available online

PLOS ONE

Regulation of Copper Homeostasis and Biotic Interactions by MicroRNA 398b in Common Bean

Loreto Naya[1◔¤], Sujay Paul[1◔], Oswaldo Valdés-López[1,2], Ana B. Mendoza-Soto[1], Bárbara Nova-Franco[1], Guadalupe Sosa-Valencia[3], José L. Reyes[3], Georgina Hernández[1]*

1 Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos. México, **2** Laboratorio de Bioquímica, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México. Tlalnepantla, Estado de México. México, **3** Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos. México

Abstract

MicroRNAs are recognized as important post-transcriptional regulators in plants. Information about the roles of miRNAs in common bean (*Phaseolus vulgaris* L.), an agronomically important legume, is yet scant. The objective of this work was to functionally characterize the conserved miRNA: miR398b and its target Cu/Zn Superoxide Dismutase 1 (*CSD1*) in common bean. We experimentally validated a novel miR398 target: the stress up-regulated *Nodulin 19* (*Nod19*). Expression analysis of miR398b and target genes –*CSD1* and *Nod19*- in bean roots, nodules and leaves, indicated their role in copper (Cu) homeostasis. In bean plants under Cu toxicity miR398b was decreased and *Nod19* and *CSD1*, that participates in reactive oxygen species (ROS) detoxification, were up-regulated. The opposite regulation was observed in Cu deficient bean plants; lower levels of *CSD1* would allow Cu delivery to essential Cu-containing proteins. Composite common bean plants with transgenic roots over-expressing miR398 showed *ca.* 20-fold higher mature miR398b and almost negligible target transcript levels as well as increased anthocyanin content and expression of Cu-stress responsive genes, when subjected to Cu deficiency. The down-regulation of miR398b with the consequent up-regulation of its targets was observed in common bean roots during the oxidative burst resulting from short-time exposure to high Cu. A similar response occurred at early stage of bean roots inoculated with *Rhizobium tropici*, where an increase in ROS was observed. In addition, the miR398b down-regulation and an increase in *CSD1* and *Nod19* were observed in bean leaves challenged with *Sclerotinia scleortiorum* fungal pathogen. Transient over-expression of miR398b in *Nicotiana benthamiana* leaves infected with *S. sclerotiorum* resulted in enhanced fungal lesions. We conclude that the miR398b-mediated up-regulation of *CSD* and *Nod19* is relevant for common bean plants to cope with oxidative stress generated in abiotic and biotic stresses.

Citation: Naya L, Paul S, Valdés-López O, Mendoza-Soto AB, Nova-Franco B, et al. (2014) Regulation of Copper Homeostasis and Biotic Interactions by MicroRNA 398b in Common Bean. PLoS ONE 9(1): e84416. doi:10.1371/journal.pone.0084416

Editor: Maeli Melotto, University of Texas, United States of America

Received September 23, 2013; **Accepted** November 19, 2013; **Published** January 6, 2014

Funding: This work was supported by Dirección General de Asuntos del Personal Académico (DGAPA)- UNAM. Grant no. PAPIIT IN202213 and Postdoctoral Scholarships to LN and SP. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding received for this study.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: gina@ccg.unam.mx

¤ Current address: Institut Jean-Pierre Bourgin, INRA Centre de Versailles-Grignon Route de St-Cyr (RD10), Versailles, France

◔ These authors contributed equally to this work.

Introduction

The small RNAs are key post-transcriptional regulators in eukaryotes; microRNAs (miRNAs) are the best-characterized subgroup. In plants miRNAs are involved in fundamental processes such as development, phytohormonal metabolism / regulation and stress response. The plant miRNA precursors, generally transcribed by RNA Polymerase II, adopt a stem-loop structure that is excised and methylated by a dicer-like 1 (DCL1) and HEN1 proteins, respectively. Mature miRNAs are exported to the cytosol and loaded into the RNA induced silencing complex (RISC). MiRNAs repress target expression by transcript excision or translation inhibition after base complementary recognition of target mRNA transcript [1,2].

During the recent years, the use of high-throughput sequencing technologies has facilitated the identification of miRNA populations and their target mRNAs from different plants including species from the legume family. Legumes are important for sustainable agriculture, as they are able to form nitrogen-fixing symbioses with rhizobia and soil-nutrient scavenging symbioses with mycorrhizal fungi. Common bean (*Phaseolus vulgaris*) is the most important crop legume for human consumption; it is the main source of proteins for people in countries of South-America and Africa. Upon infection with *Rhizobium etli* or *R. tropici* common bean roots develop determinate N_2-fixing nodules [3]. Recently our groups have used different approaches to contribute to the identification and functional characterization of *P. vulgaris* miRNAs and their targets. Arenas–Huertero et al. [4] sequenced small RNA libraries and identified several conserved and six novel miRNAs, some of these responded to drought and salinity. Valdés-López et al. [5] used a macroarray-hybridization approach to identify common bean miRNAs that responded to nutrient deficiency and manganese toxicity. Targets for common bean miRNAs have been predicted through computational analyses and

some conserved targets that showed a negative correlation of expression with specific miRNAs have been experimentally validated [4,5]. The role of miR399 in the PHR1 signaling pathway for phosphorus starvation in common bean roots has been demonstrated [6]. More recently, Peláez et al. [7] used high-throughput sequencing for the identification and characterization of *P. vulgaris* miRNAs. In this work we analyzed the role of miR398 in common bean plants under abiotic and biotic stresses.

MiR398 is conserved in several plant species including legumes such as *Medicago truncatula* [8], *Lotus japonicus* [9], soybean (*Glycine max*) [10], peanut (*Arachis hypogea*) [11], urdbean (*Vigna mungo*) [12] and common bean [4,5,7]. Its conserved targets are transcripts coding for the Copper-Zinc Superoxide Dismutases (CSD) [13]. CSDs are scavengers of ROS and are important for stress resistance and survival in plants; they can rapidly convert highly toxic O_2^- to less toxic H_2O_2. Besides *CSD1* and *CSD2*, other two *Arabidopsis thaliana* (Arabidopsis) transcripts coding for: Cox5b-1 (a subunit for the mitochondrial Cytochrome *c* Oxidase) and CCS1 (the Copper Chaperone for CSD) have been validated as miR398 targets [13,14,15]. A degradome sequencing analysis in soybean identified transcripts for *MtN19*-like (*M. truncatula* Nodulin 19-like) protein and for a serine-type endopeptidase as miR398 targets [10].

MiRNA398 was the first miRNA described as oxidative stress responsive in plants [16]. In the oxidative stress condition, generated by biotic and abiotic stresses, production of reactive oxygen species (ROS) is increased; some of these are highly toxic and must be rapidly detoxified by various cellular enzymatic and non-enzymatic mechanisms. Oxidative stress generated upon exposure to toxic concentrations of metals like copper (Cu), suppresses Arabidopsis miR398 expression that is essential for the accumulation of CSD1 and CSD2 required for ROS detoxification [16]. In addition, Arabidopsis miR398 is decreased in salt stress [17], in high light and in methyl viologen treatments [16,18]. Down-regulation of miR398 has also been observed in *Medicago sativa* and *M. truncatula* under toxic mercury, cadmium or aluminum concentrations [19,20]. Contrastingly, miR398 is up-regulated in nitrogen-deficient [21] and in heat-stressed Arabidopsis [22] as well as in drought-stressed *M. truncatula* [23]. In addition, miR398 responds to phosphate deficiency in different plant species such as Arabidopsis, common bean, soybean and tomato [5,24,25]. MiR398 is a central regulator for Cu homeostasis: its down-regulation in Cu toxicity results in high CSDs for ROS detoxification whereas in Cu deficiency increased levels of miR398 are observed together with increased Fe (iron) Superoxide Dismutase (FSD) that takes over ROS detoxification and limited Cu is delivered to Plastocyanin (PC), a Cu-containing protein that is essential for photosynthesis [15,26]. The GTAC sequence present in the Arabidopsis miR398 promoter is an important feature in Cu responsiveness. This motif is recognized by the SPL7 transcription factor that binds to the promoter and regulates the expression of miR398. In addition SPL7 regulates the expression of other Cu-deficiency responsive miRNAs: miR397, miR408 and miR857 [27]. Moreover, Arabidopsis miR398 expression is regulated by sucrose [28]. Furthermore, the levels of miR398 decrease in Arabidopsis leaves infiltrated with avirulent strains of *Pseudomonas syringae* pv. tomato while *CSD1* was up-regulated [29].

The aim of this work was to functionally characterize miR398b in common bean plants. We confirmed the *Nod19* (*Nodulin 19*) transcript as a novel target of bean miR398. We characterized the response of miR398b and its targets *CSD1* and *Nod19* in common bean plants under Cu stress. We achieved the over-expression of miR398 in transgenic roots of bean composite plants, observing a reduction of *CSD1* and *Nod19* target gene transcripts both in control and Cu-deficiency stress conditions. In addition, the response of miR398 and its targets to symbiotic and pathogenic interactions was investigated. Our work extended the knowledge of the role of miR398b in abiotic and biotic stresses in an agronomically important legume.

Results and Discussion

MiR398 isoforms and target genes

The Arabidopsis miR398 family is encoded by three loci that are transcribed and processed into the miR398a, miR398b and miR398c isoforms [13,30]. This family is highly conserved among seed plants; two and three miR398 isoforms have been detected in soybean and *M. truncatula* legume plants, respectively (www.mirbase.org). Peláez et al. [7] identified two miR398 isoforms in common bean: miR398a (20 nucleotides) and miR398b (21 nucleotides) that differ in two nucleotides and map in different loci of the *P. vulgaris* genome (www.phytozome.net, V.1.0). While miR398b was highly detected in miRNA libraries analyzed by RNA-seq, especially in seedlings and leaves, miR398a was poorly detected in all libraries [7]. In contrast to Arabidopsis miR398b and miR398c, the level of miR398a is constant in different Cu conditions tested, both in wild type and in *spl7* mutant plants lacking the SPL7 positive regulator of miR398 and Cu-responsive genes. This is consistent with the observation that the miR398a promoter does not contain GATC SPL7-DNA binding motifs [27]. Through quantitative RT-PCR (qRT-PCR) expression analysis we observed that the miR398a transcript level was very low and constant in all the tested tissues from control or Cu-stressed plants (Table S1), contrasting with our data for miR398b (see below). Therefore in this work we proceeded with the analysis of only the miR398b isoform of common bean (Phvul.008G202400.1, *P. vulgaris* genome sequence V.1.0, www.phytozome.net).

Among conserved targets of miR398, *CSD1* is the most studied [16]. *P. vulgaris CSD1* gene (Phvul.006G097000.1) presents a miR398b matching site between 5′UTR and exon 1 sequence and has been validated as a miR398 target (C. De la Rosa et al., in preparation). Through a degradome study, Song et al. [10] detected the *MtN19*-like transcript (Glyma15 g13870) as a soybean miR398 target. This is orthologous to *MtN19* first identified in *M. truncatula* together with other 28 cDNA clones (nodulins *MtN1* to *MtN29*) induced during nodule development [31]. On this basis, we did a BLAST search [32] within the common bean genome sequence (http://www.phytozome.net/search.php?method = Org_Athaliana) and found Phvul.006G127300.1 locus as the *MtN19* ortholog in common bean; this could be a target for miR398b. This gene, annotated as "stress up-regulated Nod19", is composed of three exons and 2 introns; its transcript sequence has 1418 nucleotides with 63.4% identity with *MtN19*. It encodes for a deduced 404 amino acid protein. The miR398b matching site, with a predicted score of 5.0 [13], mapped at the 5′ end of exon 1 (Fig. 1A). The 5′RLM-RACE approach was used to experimentally validate *Nod19* mRNA cleavage site. As shown in Fig. 1A, 5 out of 6 independent clones mapped the site of cleavage at the predicted position. Therefore, we demonstrated that *Nod19* is a target of common bean miR398b.

The *MtN19* gene, reported as a *M. truncatula* nodulin [31], is expressed both in young nodules and roots, but its function is still unknown [33]. *MtN19* activation is strongly reduced in the *efd-1* null deletion mutant lacking the EFD transcription factor, from the ERF family, so it has been proposed as target of this transcriptional regulator [33,34]. *MtN19-like* genes have also been

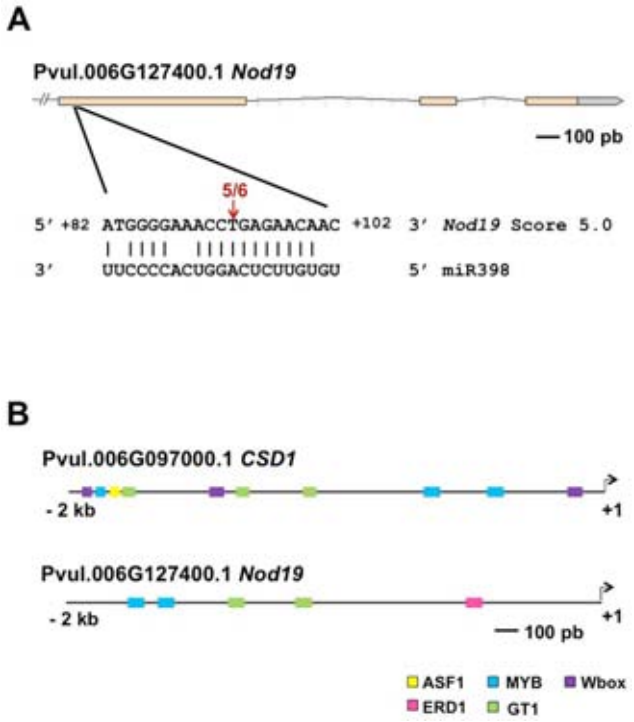


Figure 1. Common bean target genes for miR398. (**A**) *Nod19* gene structure according to the *P. vulgaris* genome sequence V.1.0 (www.phytozome.net). Exon regions are indicated with salmon-colored boxes and introns with black lines. The experimental validation of miR398 cleavage site was performed using a modified 5′ RLM-RACE assay. *Nod19* predicted target site is shown base-paired to miR398b; vertical lines indicate Watson-Crick base-pair and a space indicates a mismatch. The arrow above the *Nod19* mRNA indicates the number of independent clones that mapped the site of cleavage to that position. (**B**) Selected *cis*-elements identified in the promoter regions of *CSD1* and *Nod19* genes by PlantPan tool sequence analysis. Boxes for transcription factors DNA-binding are shown, these were color-coded as indicated.
doi:10.1371/journal.pone.0084416.g001

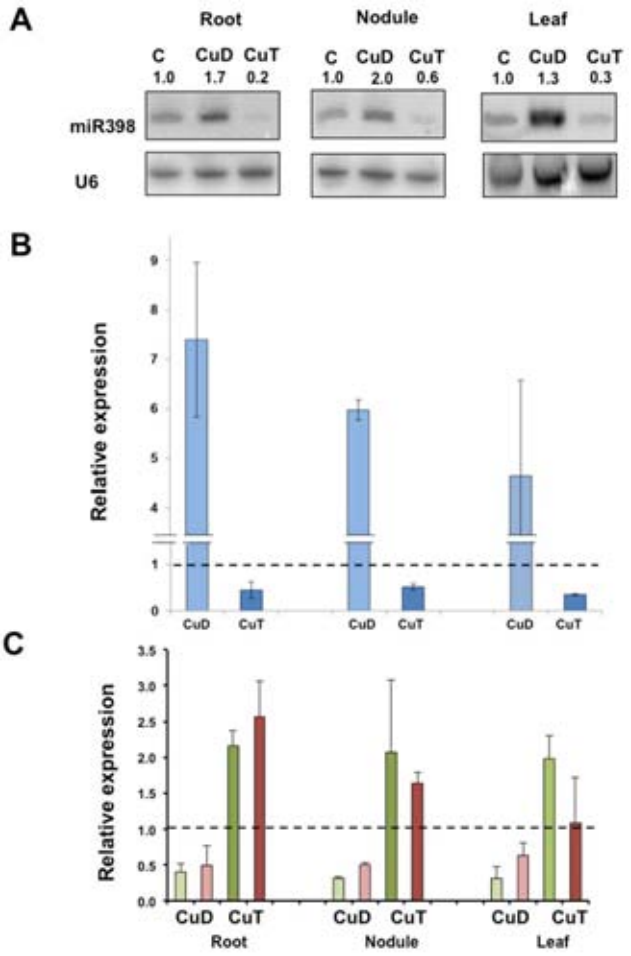


Figure 2. Expression pattern of miR398 and target genes *CSD1* and *Nod19* in tissues from common bean plants under copper deficiency (CuD) or copper toxicity (CuT). (A) miR398 levels in roots, nodules and leaves of plants grown under control (C) or stress (CuD or CuT) conditions were detected by Northern blot analysis using U6snRNA as loading control. Signal intensity of the hybridization bands was calculated and the expression ratio (stress:control) was obtained. Relative expression of **(B)** miR398b (blue) and of **(C)** target genes CSD1 (green) and Nod19 (red) in roots, nodules and leaves of plants grown under CuD (light colors) or CuT (dark colors) as determined by qRT-PCR. Values were normalized to the value from the C condition that was set to 1 as indicated with a dashed line. Values represent the average ± SD from three biological replicates.
doi:10.1371/journal.pone.0084416.g002

reported for other (monocot and dicot) plants such as Arabidopsis, rice, tomato, pea and *Phaseolus acutifolius* [35]. In addition to the regulation of *MtN19* during nodulation, it is induced in response to stress such as high light or drought stresses in Arabidopsis [36] and treatment with Bruchin B, a lipid-derived insect elicitor, in pea pods [35]. On this basis, it has been proposed that MtN19-like proteins that belong to the Stress Up-Regulated Nodulin 19 (SURNod19) family (Protein Families Database of Alignments and HMMS, pfam.sanger.ac.uk/) play important roles in plant stress responses [35]. To our knowledge *MtN19*-like transcripts have only been proposed as miR398 target in soybean [10], but not in *M. truncatula* or other plants. In this work we present evidence of the miR398b-mediated regulation of *Nod19*, together with *CSD1*, in common bean plants under abiotic and biotic stress conditions.

The validation of common bean *CSD1* and *Nod19* as miR398b targets supports their post-transcriptional regulation by this miRNA. However to gain insight into the transcriptional regulation of these genes we analyzed their promoter sequences (2 kb upstream from the initiation codon) with online PlantPan tool (http://plantpan.mbc.nctu.edu.tw/index.php). Figure 1B depicts selected *cis*-elements identified in the *CSD1* and *Nod19* promoters. In case of *CSD1* promoter three Wbox sites for WKRY transcription factor DNA-binding and one ASF-1 (abiotic and biotic stress differentially stimulated) site were found. ASF-1 site is activated by salicylic acid [37] while WKRY transcription factors activate transcription of specific genes mediated by this phytohormone [38]. The *Nod19* promoter contained an ERD1 (early responsive to dehydration) site. There are several GT-1 and MYB recognition sites in both promoters. The GT-1 cis-element interacts with GT-1-like transcription factor and is required for the induction of pathogen or NaCl-stress responsive genes in Arabidopsis and soybean [39]. Transcription factors from the MYB super-family are involved in different plant processes such as development, secondary metabolism and also in response to stresses such as salt and exogenous application of ABA [40]. On this basis, we can propose that, in addition to the post-transcriptional regulation by miR398, in common bean *CSD1* and *Nod19* gene expression might be regulated by stress-responsive transcription factors.

Response of miR398b and its target genes to copper stresses

Cu^{+2} is an essential redox-active micronutrient for plant nutrition. It participates as catalytic cofactor in multiple metabolic pathways, but it can become toxic at high concentrations both for plants and animals. Plants posses several mechanisms to finely control Cu homeostasis [41].

Cu concentrations in non-contaminated soils and sediments are usually low: 20–30 ppm or<1 μM [42,43]. Human activities such as mining, land application of sewage sludge, and discharge of untreated urban and industrial residues, led to widespread soil contamination with Cu. For example, El-Nennah et al. [44] reported 25-fold increase in Cu content in soils that had been irrigated by sewage effluents for prolonged periods (25–47 years). Cu levels in soil as high as 100-fold increased (2000 ppm) from normal levels have been recorded in mining areas and in the vicinity of Cu smelters [42]. Such high Cu concentrations are toxic and result in deleterious effects that reduce plant growth and crop productivity. Deficiency or low Cu bioavailability in soil also affects plant productivity and reduces the nutritional value of crops, thus affecting human food. For example, Cu soil concentration of 0.7 – 2.5 ppm led to abnormal growth of Douglas fir plants in the Netherlands [45].

The role of miR398 in Cu homeostasis has been previously described for Arabidopsis and other plants [16,26]. In this work we assessed whether common bean miR398b has a similar role. We analyzed miR398b and target genes (*CSD1* and *Nod19*) expression in bean plants under Cu toxicity (CuT) or Cu deficiency (CuD) as compared to control plants growing in nutrient sufficiency. For growth of common bean plants in control and stress conditions we used a hydrponic system previously described [5]. For CuT condition the plant solution contained 70 μM $CuSO_4$, equivalent to 70-fold increase as compared to the Cu level in control condition; while Cu-deprived media was used for CuD condition. Similar Cu levels have been reported for Cu toxicity studies in common bean expanding leaves or seedlings [46,47]. The Cu-fold increase used for CuT is similar to that reported in Cu-contaminated soils [42]. The stress induced by each treatment was confirmed by the observation of characteristic visual symptoms and by the induction of marker genes that was verified in each experiment. For plants under CuT treatment the expression of the *Cytosolic Ascorbate Peroxidase* (*APX*, Phvul.011G071300) marker gene [48] was determined and *FSD* (Phvul.007G135400.1) expression [41] was determined for CuD plants. Plantlets adapted to hydroponic growth conditions were inoculated with *Rhizobium tropici*, when functional nodules were formed [5], control plants were kept growing in nutrient-full media, or changed to CuT or CuD media. After 7 days the roots, nodules and leaves from control or stressed plants were harvested for gene expression analysis (Fig. 2). We used the Northern blot approach to determine the miR398 expression in root, nodules and leaves of Cu-stressed and control bean plants. A miR398b probe was used for blot hybridization but the signals observed might reflect the combined levels of miR398b and miR398a isoforms whose sequence only differs in 2 nucleotides [7]. Similar results were obtained for the three tissues analyzed (Fig. 2A). In CuD treatment the miR398 level increased in roots, nodules and leaves as compared to levels from control plants, whereas it was almost undetectable in all the CuT-treated tissues (Fig. 2A). We used the qRT-PCR approach and a miR398b specific primer, as another, more sensitive, alternative method for the validation of the expression pattern of miR398b in control vs. Cu-stressed tissues (Fig. 2B). As compared to control conditions, in CuD the miR398b levels were increased *ca.* 7.5- 6- and 4.5-fold in root, nodules and leaves, respectively, while they were almost negligible in CuT plants (Fig. 2B). Though a similar tendency, up- or down-regulation, was observed in the two methods used, there was a variation among expression ratios (Cu-stress/control) calculated from Northern blots as compared to those from qRT-PCR analyses (Fig. 2A and B). This could be attributable to different sensitivities of the two methods and also different specificity since in Northern blot analysis we are detecting miR398a/b isoforms. The transcript levels of the *CSD1* and *Nod19* target genes in roots, nodules and leaves from control and Cu-stressed plants were determined by qRT-PCR (Fig. 2C). The expression of both target genes showed a negative correlation with miR398b expression in all the tissues and in both stress conditions (Fig. 2C). As compared to control conditions, *CSD1* and *Nod19* expression levels were decreased in CuD-stressed roots, nodules and leaves, thus indicating the miR398b-induced mRNA target cleavage (Fig. 2C). Whereas, an evident up-regulation of target genes was observed in CuT stressed tissues, except for *Nod19* in leaves (Fig. 2C).

The rapid increase in ROS concentration is called "oxidative burst"; this is better characterized when produced as a defense response to pathogen attack though it also occurs in response to abiotic stresses such as nutrient toxicity / deficiency, drought, heat stress and metal toxicity [49,50]. Under CuT ROS are produced by autoxidation and Fenton reaction [51]. Sgherri et al. [52] reported the analysis of the early production -from 15 min to 6 h- of activated oxygen species in root apoplast of wheat following Cu excess. Also, Cuypers et al. [48] analyzed the ROS production and metabolic response of *P. vulgaris* leaves during early stages -up to 48 h- of exposure to high Cu. In this work we analyzed the response of miR398b and its target genes to the oxidative burst resulting from exposure of common bean roots to high Cu (Fig. 3). Plants were grown in hydroponic system with nutrient solution containing 70 μM $CuSO_4$ and roots were harvested from 0 to 48 h after treatment. ROS content in root tips was analyzed after 2′,7′- dichlorodihydrofluorescein diacetate (H_2DCF-DA) incubation and subsequently observed by fluorescence microscopy. A significant increase in fluorescence intensity was observed 12 h, 24 and 48 h after Cu exposure, showing a 10-fold peak at 12 h (Fig. 3A). The transcript levels of miR398b and its targets were analyzed by qRT-PCR. The level of mature miR398b showed a significant decrease at 48 h after Cu application (Fig. 3B). MiR398b differential expression inversely correlated with that of its target genes. A *ca.* 2-fold increase in *CSD1* transcript was detected after 24 h and 48 h in CuT and a minor increase (*ca.* 1.5-fold) was detected for *Nod19* transcript (Fig. 3C).

We conclude that miR398b is involved in common bean Cu homeostasis, similar to what is known for other plants [16,26]. CuT stress is ascribed to stimulated generation of ROS that modify the antioxidant defense and elicit oxidative stress, both at late and early (oxidative burst) stages of metal exposure [48,52,53]. The suppression of miR398b expression in common bean roots, nodules and leaves at long period of CuT and in roots at early stages is important for the increase of *CSD1* transcript (Figs. 2 and 3) resulting in the accumulation of this enzyme important for ROS detoxification and oxidative stress tolerance. *Nod19* transcript was slightly increased mainly in roots and nodules after long Cu exposure (Fig. 2), suggesting that this protein may play a role in the oxidative stress response of common bean; however its function has not been described.

Cu is an essential element in plants, when limited in soils it provokes symptoms that affect the yield and nutritional value of crops. It participates as a redox catalytic cofactor in multiple proteins including cytochrome *c* oxidase, CSD and PC. While

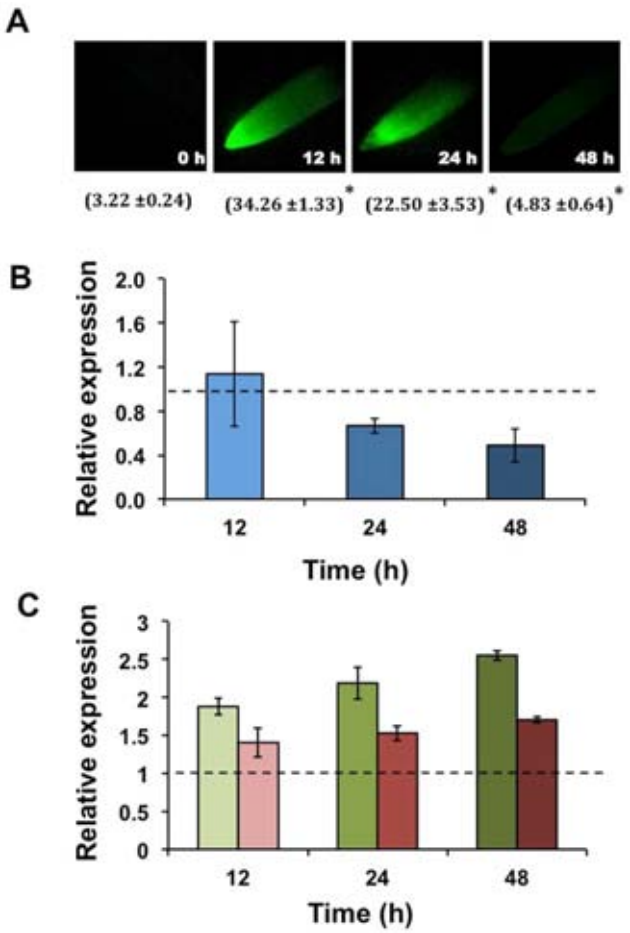


Figure 3. Reactive oxygen species (ROS) content and expression pattern of miR398 and target genes *CSD1* and *Nod19* in roots exposed to high Cu (CuT). Measurements were done at initial time (0 h) and after 12, 24 and 48 h of high Cu (70 μM $CuSO_4$) application. (A) Histological (fluorescence) detection of ROS accumulation in CuT stressed root tips using 2′,7′- dichlorodihydrofluorescein diacetate (H_2DCF-DA). The values in parenthesis indicate the average integrated fluorescence intensity per unit area of root tissue ±SD. Asterisk: Student's *t* test, $P \leq 0.05$. Relative expression, determined by qRT-PCR, of (B) miR398b (blue) and of (C) target genes *CSD1* (green) and *Nod19* (red) in CuT-stressed roots at the indicated time points. Values were normalized to the value from the C condition that was set to 1 as indicated with a dashed line. Values represent the average ± SD from three biological replicates.
doi:10.1371/journal.pone.0084416.g003

CSDs are dispensable for ROS detoxification since they can be replaced by FSDs, PC is essential for the photosynthetic electron flow in higher plants [26]. In Arabidopsis miR398 increases in Cu-starvation and it is involved in the mechanism to regulate Cu-containing proteins [26,54]; our data indicate that a similar mechanism holds for Cu-deprived common bean plants. The levels of miR398 highly increased in roots, nodules and leaves of CuD bean plants lead to very low level of *CSD1* (Fig. 2) that would prioritize the delivery of limited Cu to PC.

Over-expression of miR398 in composite plants

The study of transgenic plants with over-expression or inactivation of miRNA has allowed to gain insight or to demonstrate the regulatory functions of specific miRNAs. For example, Li et al. [55] reported the study of Arabidopsis transgenic plants over-expressing miR398b, miR160a, miR773 or miR158a that led to demonstrate the role of these miRNAs in the regulation of pathogen-associated molecular pattern-triggered plant innate immunity.

In this work we aimed to modulate the expression of miR398b to further study the role of this miRNA in common bean. In contrast to Arabidopsis, common bean and other legumes are not amenable to stable genetic transformation, and hence, protocols for high-throughput generation of transgenic legume plants are not available. The efficient protocol for *Agrobacterium rhizogenes*-mediated bean transformation to generate "composite plants" with transgenic roots and un-transformed aerial organs has been used as an alternative for stable transformation in common bean and other recalcitrant species [6,56]. In this work we aimed to use this protocol in conjunction with constructs for over-expression or inactivation of miR398b. For miR398 inactivation we proposed to use the target mimicry strategy [57]. The MIM398 construct, with *Pv4* (*IPS1*) backbone, was designed to give rise to mimicry transcripts that specifically trap mature miR398 thus hindering miR398 activity. The OE398 construct contained the 35SCaMV promoter fused to the miR398c precursor from *M. truncatula*. Both constructs as well as the control, empty vector (EV), have the tdTomato (red fluorescent protein, RFP) reporter gene. First, the correct plant cell expression of the transgenes from each construct was tested in *Nicotiana benthamiana* leaves previously infiltrated with *Agrobacterium tumefaciens* LBA4404 bearing the corresponding plasmid. After verifying the transgene expression (data not shown) each plasmid was introduced into *A. rhizogenes* K599 and used for common bean genetic transformation and generation of composite plants [56].

A satisfactory transformation frequency (70 – 80%) was obtained with EV or OE398 plasmids. However with MIM398 plasmid, an unexpected low transformation frequency (≤20%) was obtained repeatedly in at least four independent experiments. Besides, among plants that developed hairy roots after A. *rhizogenes* / MIM398 infection only a few transgenic roots expressed RFP indicating diminished co-transformation with MIM398 binary vector. This problem was not observed for other MIM construct tested by our group at the same time nor have been reported by other groups. We hypothesize that the MIM inactivation of miR398 could affect the interaction / infection of the *A. rhizogenes* pathogen or could interfere with root development, or both (as discussed below).

We followed the analysis of common bean composite plants showing miR398b over-expression. Throughout this work we obtained several composite plants with transgenic roots bearing EV or OE398 construct. Each transgenic root results from a different transformation event and therefore each individual root may show a different degree of miR398 overexpression. Table 1 illustrates this phenomenon; the miR398 normalized expression of four representative individual OE398 transgenic roots from different composite plants varies from 3- to 9-fold as compared to expression in EV control roots. The miR398 overexpression values correlate with decreased expression of *CSD1* target gene in OE398 transgenic roots (Table 1). These results indicate that the *M. truncatula* miR398c precursor from the OE398 construct is highly transcribed and adequately processed in common bean transgenic roots. Increased transcript levels were also observed in nodules of OE398 composite plants inoculated with *R. tropici*. However, nodulation and nitrogenase activity (determined by acetylene reduction assay) was similar in OE398 and in EV inoculated composite plants thus indicating that the over-expression of miR398b had no effect in the bean-rhizobia symbiosis.

Table 1. Expression of miR398 and *CSD1* in individual OE398 transgenic roots.

	Expression ratio (OE398/EV)	
	miR398	***CSD1***
HR1	6	0.24
HR2	3.2	0.36
HR3	4.3	0.29
HR4	9.3	0.41

Northern blot analysis was performed using specific probes; U6 snRNA was used as loading control. Signal intensity of the hybridization bands was determined and the miR398 or *CSD1* expression ratio in OE398 as compared to control (EV) individual transgenic roots was obtained.
doi:10.1371/journal.pone.0084416.t001

We performed a comparative analysis of composite bean plants showing miR398b over-expression vs. EV composite plants grown in nutrient sufficient (control, C) or in CuD conditions, for 7 days. This experiment would allow knowing if miR398b over-expression is observed only in C growth conditions (Table 1) or also in CuD transgenic roots and if such alteration in miRNA expression would result in a much lower level of its target genes. Results are shown in Figure 4; miR398b, *CSD1* and *Nod19* transcript levels were determined by qRT-PCR from individual transgenic roots derived from biological replicates of composite plants. In C condition, the OE398 composite plant showed a 3-fold miR398 over-expression (Fig. 4A). In agreement with data presented in Fig. 2 for un-transformed plants, in CuD condition the EV composite plant showed a high endogenous miR398b induction (Fig. 4A) and a consequent decrease in *CSD1* and *Nod19* transcript levels (Fig. 4B). However the CuD-stressed OE398 composite plant showed a much higher miR398 transcript level, interpreted as the combination of over-expression and CuD response (Fig. 4A). Consequently, a very low almost undetectable level of *CSD1* and *Nod19* transcript were observed in CuD-stressed OE398 common bean (Fig. 4B). We then asked if the almost negligible level of *CSD1* and *NOD19* from OE398 transgenic roots would affect the plant response to CuD stress. For such phenotypic analysis of EV vs. OE398 transgenic roots from composite plants we determined anthocyanin content and the regulation of the expression of CuD responsive genes: *FSD*, a high affinity Cu transporters (*COPT*) and a ferric-chelate reductase (*FRO*). An increase in anthocyanin contents was observed in the crown of the root of both EV and OE398 CuD stressed plants (Fig. 4C). The accumulation of anthocyanin pigments in vegetative tissues is a hallmark of plant stress [58]. A variety of nutrient deficiencies in plants are characterized by the accumulation of flavonoids, notable red/purple colored anthocyanins, this has been well characterized in tomato leaves [59]. As mentioned before, in higher plants the abundant CSD is replaced by the Fe counterpart (FSD) upon Cu limitation, allowing plants to economize Cu when scarce, for essential chloroplastic PC [41]. In agreement, EV common bean roots from plants under CuD showed *FSD* induction (Fig. 4D). The conserved *CTR* gene family encoding high affinity Cu transporters (*COPT*) plays essential roles in Cu acquisition when this metal is limited; in Arabidopsis several members of this family (composed by 5 genes) are induced upon Cu starvation [41,60]. A common bean *COPT* gene (Phvul.011G060400) was slightly up-regulated in EV transgenic roots subjected to CuD (Fig. 4D). The *FRO* genes encode for ferric-chelate reductase that is required in most plants to acquire Fe, by releasing Fe from organic compounds; several genes from this family are induced upon Fe limitation [61]. The enzyme encoded by Arabidopsis *FRO3* gene is involved in the reduction of divalent Cu to monovalent Cu and so, beside its regulation in Fe-deficiency, its expression is elevated in Cu-limited roots and shoots [62]. We determined the expression of a common bean *FRO* gene (Phvul.006G142300) in transgenic roots of composite plants under CuD stress and C conditions and observed an up-regulation in EV roots (Fig. 4D). In Arabidopsis induction of CuD responsive genes such as *FSD*, *COPT* and *FRO* as well as miR398, is positively regulated by *SPL7* that binds to GTAC motifs present in these genes' promoters [27], similar gene regulation might be occurring in common bean giving rise to the expected up-regulation response of the CuD responsive genes analyzed in EV roots under Cu deficiency (Fig 4A). Similarly, we measured the transcript level of *FSD*, *COPT* and *FRO* in OE398 roots from composite plants grown in control and CuD conditions (Fig. 4D). When comparing the CuD responsive gene expression ratios in EV vs. OE398 roots a similar trend was observed, albeit with a diminished up-regulation in OE398 composite plants indicating a decreased CuD gene response in roots with very low *CSD1* resulting from the miR398 over-expression. We suggest that the almost negligible amount of *CSD1* in CuD transgenic roots over-expressing miR398 (Figs. 4A, B) would allow to spare more Cu for its delivery to other essential Cu-containing proteins, as compared in EV roots, under Cu deficiency. In this situation the OE398 composite plants would be sensing less Cu starvation as compared to EV plants and their CuD-genes response would be diminished.

Response of miR398 and its target genes to symbiotic and pathogenic interactions

Arabidopsis miR398 is regulated during biotic interactions with an avirulent strain of *P. syringae* pv. tomato [29]. In this work we assessed the regulation of common bean miR398b in biotic interactions, considering both a symbiont and a pathogen. It has been proposed that plant symbiosis and pathogenesis are variations on a common theme [63,64]. The common strategies that guide the interplay between symbiotic and pathogenic plant partners include: induction of enzymes of the phenylpropanoid pathway for the synthesis of end products (flavonoids, isoflavonoids, phytoalexins) that play diverse roles in plant-biotic interactions, the hypersensitive response that entails ROS (mainly H_2O_2) production and induction of peroxidases as well as changes in the intracellular Ca^{+2} concentration [63,64]. Previous works have indicated that rhizobia might be recognized as intruders that somehow evade or overcome the plant defense response [63–66].

a) Interaction with *Rhizobium tropici*. There is increasing evidence that ROS play important roles, perhaps related to signaling, in the establishment as well as in the early and later stages of the legume-rhizobia symbiosis [65,66]. Increasing and transient ROS levels have been detected as early as seconds and up to 3 min after addition of Nod factors (NF, lipochitooligosaccharides signals secreted by rhizobia and perceived by legume roots) in common bean root hairs. This response seems to be characteristic of the symbiotic interaction since upon chitosan fungal elicitor induced a different response showing sustained increasing ROS signal [65]. In *M. truncatula* and *M. sativa* ROS production in infection threads, roots, and nodules primordia was observed from 12 to 60 h after inoculation with *Sinorhizobium meliloti* or treatment with NF [67,68]. Accumulation of ROS in early symbiosis stages depended upon production of compatible NF by the bacteria and functional NF perception by the plant and it showed a similar pattern to the expression of an early nodulin encoding a peroxidase protein [68].

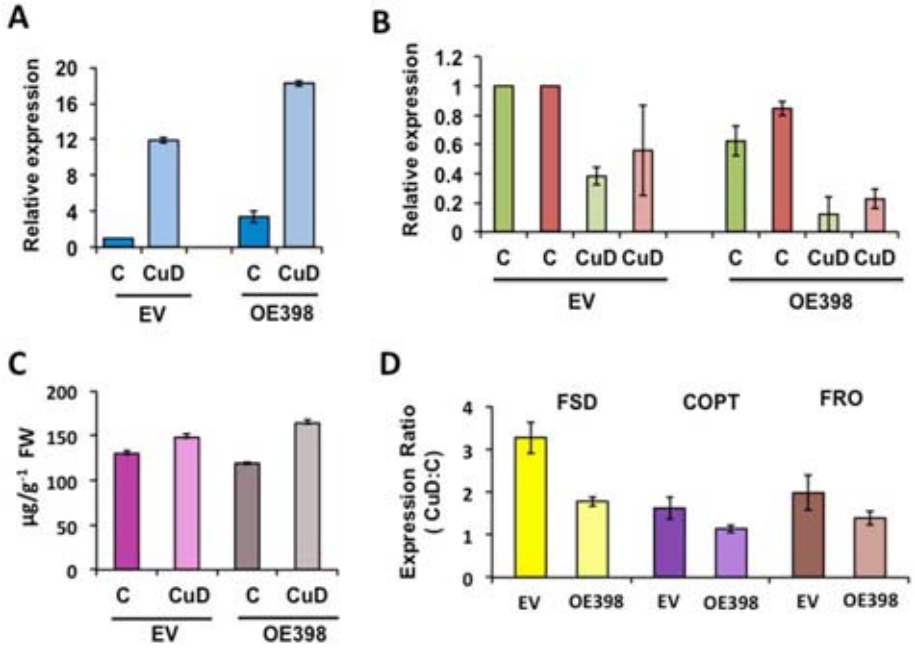


Figure 4. Effect of miR398b over-expression in transgenic roots from composite plants grown under CuD. Composite plants were obtained through *A. rhizogenes* transformation with EV or with OE398 plasmid, these were grown in control (sufficient nutrient) condition (C) or in CuD stress condition. (**A**) Relative expression of miR398b (blue) and of (**B**) target genes *CSD1* (green) and *Nod19* (red) determined by qRT-PCR; values were normalized to the value from the EV roots grown in the C condition that was set to 1. (**C**) Anthocyanin contents in root crown of composite plants. (**D**) Expression ratio (CuD:C) of copper-stress responsive genes: Fe superoxide dismutase (FSD, yellow), high affinity Cu transporter (COPT, purple) and ferric chelate reductase (FRO, brown). Values represent the average ± SD from three biological replicates.
doi:10.1371/journal.pone.0084416.g004

On this basis, we analyzed the regulation of miR398b and its target genes in the early stages of the common bean-rhizobia symbiosis. Plants were inoculated with R*hizobium tropici* CIAT899 and incubated in hydroponic system up to 48 h and roots were harvested at different time point to check the ROS production as well as to analyze miR398b and target genes expression (Fig. 5). Quantification of H_2DCF-DA/ROS fluorescent complexes indicated significant ROS accumulation in roots at every time point analyzed. ROS content increased *ca.* 10-fold at 3 h to 12 h post-inoculation and it decreased at 24 h and 48 h to *ca.* 5-fold as compared to bean basal levels found in bean roots prior to rhizobia inoculation (Fig. 5A). Levels of mature miR398b decreased at early stages *R. tropici* inoculation up to *ca.* 40% at 48 h (Fig. 5B). Consequently, an increase of target genes transcripts was detected with a maximum of 3-fold for *CSD1* and 2-fold for *Nod19* at 48 h post inoculation (Fig. 5C). Results suggested that miR398b repression is important to increase *CSD1* and *Nod19* content and these could play important roles for ROS regulation in the common bean early response to rhizobia inoculation.

b) Interaction with Sclerotinia sclerotiorum. The plants defense response to pathogens involves rapid changes in gene, hormone and metabolite levels; miRNAs are also part of such defense mechanisms. Several miRNAs were up-regulated while others were down-regulated in Arabidopsis leaves challenged with virulent and avirulent *Pseudomonas syringae* pv. tomato strains [69]. MiR398 was the first miRNA reported to be down-regulated in response to biotic stress in Arabidopsis leaves infiltrated with avirulent strain *P. syringae* pv. tomato DC3000 [29]. In this study, *CSD1* target gene showed increased levels and therefore a negative correlation with miR398 [29]. ROS are rapidly produced in plants as a defense response to pathogen attack, a process called oxidative burst [50]. The increased CSD1 levels were likely to detoxify ROS caused by pathogen infection and support that miR398 modulated pathogen resistance in Arabidopsis. In this work we assessed miR398b regulation in common bean pathogenic interaction. This was based on Arabidopsis knowledge [29] and also in our observation of impairment of infection and hairy root formation when *A. rhizogenes* with the MIM398 construct was used. We hypothesize that such impairment in a pathogenic interaction (*A. rhizogenes* – common bean) might be related to the role of miR398 and its targets.

Sclerotinia sclerotiorum, also known as white mold, is an economically important necrotrophic fungal pathogen with a broad host range [70]. *S. sclerotiorum* utilizes controlled generation of ROS for successful colonization [71,72]. CSD, besides its role in the Cu homeostasis, plays an important role in the detoxification of ROS [49]. On this basis, we tested if common bean miR398b plays a role in *S. sclerotiorum* colonization. *P. vulgaris* is susceptible to *S. sclerotiorum* infection, the characteristic fungal lesion was clearly observed in fungi colonized leaves (Fig. 6A). The accumulation of miR398b and the expression of its two target genes (*CSD1* and *NOD19*) was evaluated by qRT-PCR in common bean leaves infected with *S. sclerotiorum*. The level of miR398b was significantly reduced in common bean leaves after 48 h of infection with *S. sclerotiorum* (Fig. 6A). In contrast, expression of *CSD1* was up-regulated in the same infected leaves (Fig. 6B). Something similar was observed in the expression of *Nod19*, however, the up-regulation of this gene upon *S. sclerotiorum* infection was lower than the induction levels observed in *CSD1* (Fig. 6B). *S. sclerotiorum* can initially suppress host oxidative burst to aid infection establishment, but later promotes ROS generation as proliferation advances [73]. The expression pattern of miR398b and its targets

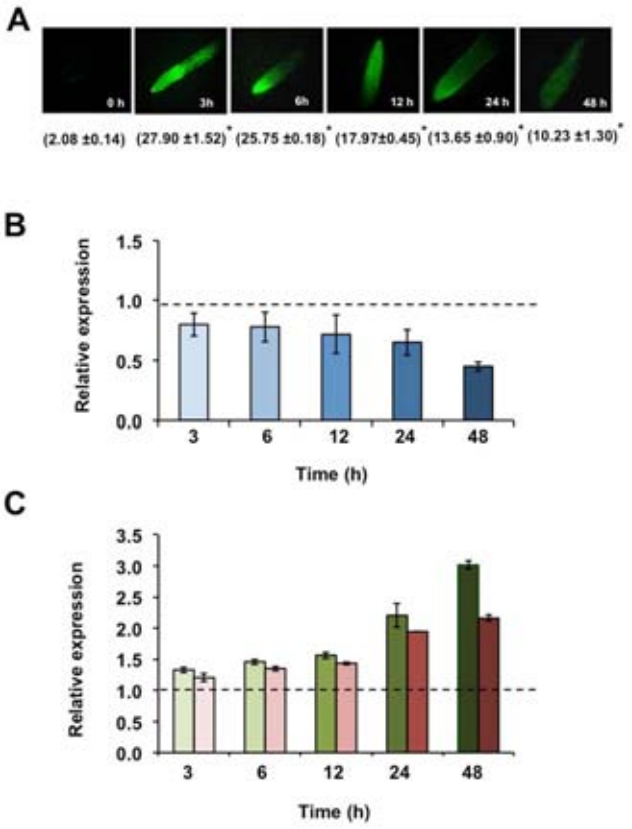


Figure 5. ROS content and expression pattern of miR398 and target genes *CSD1* and *Nod19* in roots inoculated with *Rhizobium tropici*. Measurements were done at initial time (0 h) and 3, 6, 12, 24 and 48 h after inoculation with *R. tropici*. (A) Histological (fluorescence) detection of ROS accumulation in inoculated root tips using 2′,7′- dichlorodihydrofluorescein diacetate (H_2DCF-DA). The values in parenthesis indicate the average integrated fluorescence intensity per unit area of root tissue ±SD. Asterisk: Student's *t* test, $P \leq 0.05$. Relative expression, determined by qRT-PCR, of (B) miR398b (blue) and of (C) target genes *CSD1* (green) and *Nod19* (red) in inoculated roots at the indicated time points. Values were normalized to the value from the C condition that was set to 1 as indicated with a dashed line. Values represent the average ± SD from three biological replicates.
doi:10.1371/journal.pone.0084416.g005

observed in this study (Fig. 6) might reflect part of the plant defense response against this fungal pathogen.

Our expression analysis suggests the participation of miR398b and its targets in the infection process of *S. sclerotiorum*. In order to prove this, the precursor of miR398 was transiently over-expressed in *Nicotiana benthamiana*. Leaves infiltrated with the OE398 construct showed a 3-fold accumulation of miR398 than non-infiltrated infiltrated leaves -showing basal accumulation of endogenous *N. benthamiana* miR398- or leaves infiltrated with EV (Fig. 7A). Interestingly infiltrated leaves over-expressing miR398b were more susceptible to *S. sclerotiorum* infection which was reflected in size of lesion (Fig. 7B, C). The accumulation of miR398b remained 48 h after *S. sclerotiorum* inoculation in OE398 infiltrated leaves as compared with non-inoculated or EV inoculated leaves (Fig. 7D).

We showed that miR398 is involved in the colonization process of a symbiont (rhizobia) and of a necrotrophic pathogen. The fast and drastic increase in ROS production in common bean roots at early stages of rhizobia inoculation is in agreement with what was reported by Cárdenas et al. [65] and by Santos et al. [67] and Ramu et al. [68] for *M. truncatula* after rhizobia inoculation or NF treatment. This phenomenon has been referred to as oxidative burst, first described for pathogenic interactions and also for symbiotic interactions such as the legume-rhizobia [50,63,65]. We interpret that the increase in *CSD1* expression observed in the symbiotic and pathogenic common bean interactions (Figs. 5 and 6) is relevant for ROS detoxification during the oxidative burst. *Nod19* expression was also increased in these biotic interactions, though to a minor level (Figs. 5 and 7). MtN19-like from pea increases in pods treated with the insect elicitor Bruchin B [35] and thus it has been proposed that this protein from the Stress Up-Regulated Nodulin 19 (SURNod19) family plays a role in plant pathogenic and stress responses. Our finding support this proposal for common bean, though the specific function of MtN19 and orthologous proteins remain to be elucidated.

Conclusions

In this work we performed a functional analysis of miR398b and its targets to elucidate their roles in Cu homeostasis and biotic stress in common bean.

We experimentally demonstrated *Nod19*, the common bean *MtN19* ortholog, as a miR398b target.

The role of miR398b in Cu homeostasis was evidenced through the expression analysis of this miRNA and its targets (*CSD1* and *Nod19*) in tissues of common bean plant subjected to CuT or CuD stresses. Low *CSD1*, mediated by high miR398b levels, in CuD bean tissues would spare limiting Cu for other Cu-containing proteins essential for plant processes. While high *CSD1*, correlating with miR398b down-regulation, would be relevant for detoxifying ROS produced in common bean plants under CuT. A similar response was observed in common bean during the oxidative burst generated by short-period exposure to high Cu.

High miR398b over-expression was achieved in transgenic roots from common bean composite plants that nearly lacked *CSD1* mRNA when stressed by CuD. This would provide less Cu limitation as compared to that in CuD EV composite plants that showed higher induction of CuD responsive genes (*FSD, COPT, FRO*) than OE398 plants.

We report the response of miR398b to rhizobial symbiotic and fungal pathogenic interactions. MiR398 was diminished in bean roots colonized by these microorganisms. The resulting increase in *CSD1* might be related to the oxidative burst produced in such interactions. *N. benthamiana* leaves with transient over-expression of miR398 were more susceptible to *S. sclerotiorum* infection. *Nod19* target gene expression also increased in roots colonized with rhizobia or *S. sclerotiorum*, something that might indicate its role in pathogenic interactions, though the function of Nod19 protein remains to be elucidated.

This work contributes to increase the knowledge of the roles of miRNAs in common bean, an agronomically important crop legume.

Materials and Methods

Plant material and growth conditions

Seeds of *Phaseolus vulgaris* Mesoamerican "Negro Jamapa 81" cultivar were surface sterilized and germinated for 2 days at 26–28 °C in darkness. Plants were grown in hydroponic system under controlled environmental conditions as previously described [5] The hydroponic trays contained 8 L of nutrient solution [74] at pH 6.5; the volume and pH were controlled along the experiment. For symbiotic conditions, N-free nutrient solution was used and

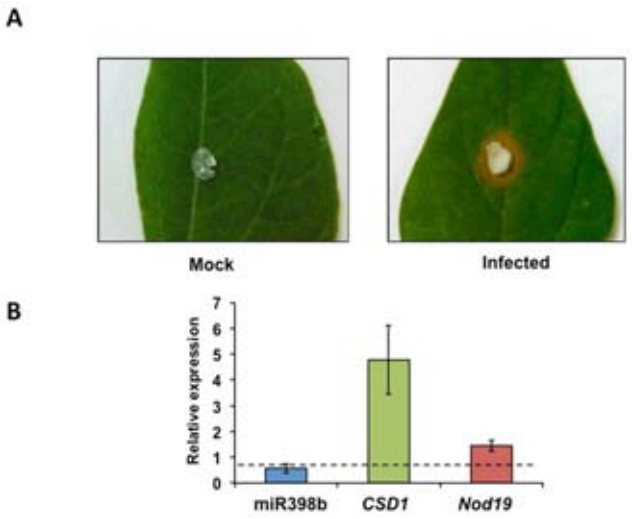


Figure 6. Expression pattern of miR398b and target genes *CSD1* and *Nod19* in common bean leaves infected with *Sclerotinia sclerotiorum*. (A) Mock (left) or *S. sclerotiorum* infected (right) common bean leaves after 24 h. (B) Relative expression of miR398b (blue) and of target genes *CSD1* (green) and *Nod19* (red) determined by qRT-PCR; values were normalized to the value from mock that was set to 1 as indicated with a dashed line.
doi:10.1371/journal.pone.0084416.g006

7d-old plants were inoculated with 10 mL of saturated liquid culture of *Rhizobium tropici* CIAT899. After 14d post inoculation when bean plants have developed small nodules actively fixing atmospheric N_2, stress was applied. For Cu toxicity (CuT) the nutrient solution was supplemented with 70 μM $CuSO_4$ and for Cu deficiency (CuD) the nutrient solution was deprived of Cu, for control condition the nutrient solution with 1 μM CuSO4 was maintained. Under the Cu-stress conditions used plants presented characteristic visual symptoms. In each CuT experiment the expression of *APX* (Phvul.011G071300), marker gene for CuT [48], was determined by qRT-PCR (see below). In each CuD experiment, the expression of *FSD* (Phvul.007G135400.1), marker gene for CuD [41], was determined. Increased expression of the marker genes indicated the stress-nature of the treatment used (data not shown). Roots, mature nodules or leaves from inoculated plants were harvested at 7d post stress. For analysis of roots at early stages of rhizobia infection, plants were inoculated as described and roots were collected at 0h, 3h, 6 h, 12 h, 24 h and 48 h post inoculation. For non-symbiotic conditions plants were grown in full-nutrient solution and the same $CuSO_4$ concentration was used for CuT condition, roots were collected at 12 h, 24 h and 48 h.

Common bean composite plants with transgenic roots [56] were generated as described below and were grown in similar CuD or control conditions as those described for un-transformed plants. Plants were analyzed at 7d post stress. Total monomeric anthocyanin contents were measured by pH differential method using a spectrophotometer. Briefly, two different liquid extracts of the samples (crown portion of the main root) were prepared using potassium chloride buffer, pH 1.0 and sodium acetate buffer, pH 4.5 and the absorbance was measured at $\lambda_{vis\text{-}max}$ and 700 nm respectively. Finally, total monomeric anthocyanin contents were determined using the absorbance values and standard formula as

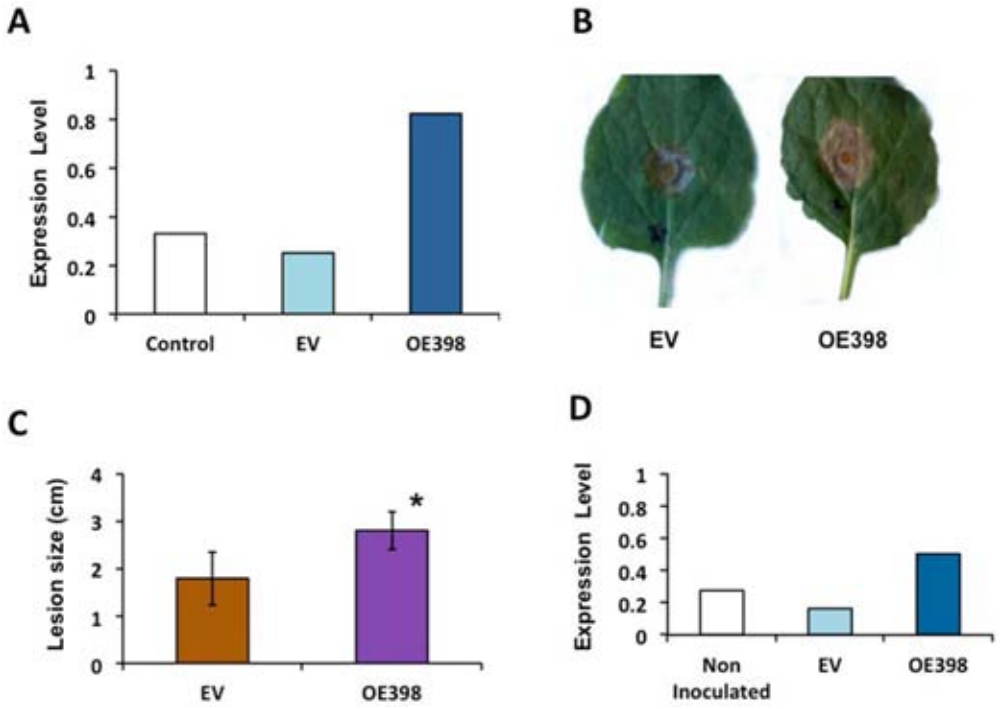


Figure 7. Effect of miR398b transient over-expression in *Nicotiana benthamiana* leaves infected with *Sclerotinia sclerotiorum*. *N. benthamiana* leaves were infiltrated with water (Control) or with *A. tumefaciens* bearing EV or OE398 plasmids and miR398b expression level was determined 3d after infiltration **(A)**. Subsequently, infiltrated leaves (EV or OE398) were inoculated with *S. sclerotiorum*. Characteristic fungal lesions **(B)** quantified by measuring the infection halo; asterisk: Student's *t* test, $P \leq 0.01$ **(C)** and miR398b expression levels determined by qRT-PCR **(D)** at 48 h after fungal infection.
doi:10.1371/journal.pone.0084416.g007

described before [75]. Transgenic roots were harvested for gene expression analysis through qRT-PCR.

All harvested tissue samples were immediately frozen in liquid N_2 and preserved at -80°C until used for RNA isolation.

ROS detection

Intracellular production of reactive oxygen species (ROS) was measured by treating the roots with 15 µM 2′,7′- dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes, Leiden, The Netherlands). Briefly, the roots were first washed gently with water and then left in the dye (15 µM H_2DCF-DA) for 10–15 min under vacuumed chamber (in dark). After incubation roots were washed twice with phosphate buffer (pH 7). Fluorescence was observed at 488 nm excitation and 530 nm emission wavelengths using a fluorescence optical microscope Axioskop 2 (Zeiss). H_2DCF-DA/ROS complexes present in the roots of bean plants were quantified based on fluorescence intensity using the NIH IMAGEJ software program (http://rsbweb.nih.gov/ij/).

DNA sequence analysis of cis-elements

The miR398 common bean target genes considered for this analysis and their corresponding ID from the *Phaseolus vulgaris* genome sequence database available in Phytozome (www.phytozome.net, V1.0) [76], are: *Cooper/Zinc Superoxide Dismutase 1* (*CSD1*) (Phvul.006G097000.1) and *Nodulin 19* (*Nod19*) (Phvul.006G127400.1). Each downloaded promoter sequence, defined as 2 kb upstream region from the initiation codon, was inspected with Plant Promoter Analysis Navigator (PlantPAN) tool (http://plantpan.mbc.nctu.edu.tw/index.php), which identifies transcription factor binding sites in a group of gene promoters [77].

RNA isolation

Total RNA was extracted from 1–2 g of frozen roots, leaves and nodules of bean plants using LiCl precipitation method or Trizol reagent (Invitrogen) according to the manufacturer's instruction. Integrity and quantification of RNA were checked by agarose gel electrophoresis and by absorbance measurements using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) respectively.

Target validation by 5′RACE

To experimentally validate the cleavage site of Nod19 target transcript we used a modified 5′ RLM-RACE approach. Total RNA (1 µg) isolated from Cu-stressed roots was subjected to a 5′RACE reaction using FirstChoice RLM-RACE kit (Ambion) omitting calf intestine alkaline phosphatase and tobacco acid pyrophosphatase treatments. Two reverse specific primers were designed downstream of miR398 cleavage site (outer primer: 5′-GTTTCAGATCCAAGCCCAAA-3′; inner primer: 5′-GGGACACATTTTTAGGTTGG-3′). The PCR reaction and cycling conditions were setup following the manufacture's protocol. Annealing temperatures were adjusted for specific primers. Finally, the nested PCR products were cloned into pCR2.1 TOPO vector (Invitrogen) and sequenced.

RNA gel blot analysis

Total RNA (20 µg) was separated in 15% acrylamide-7 M urea gel electrophoresis and transferred to a Hybond NX membrane (GE, Amersham) and then UV cross-linked twice. A synthetic DNA oligonucloetide with antisense sequence corresponding to miR398 (5′ CAGGGGCGACCTGAGAACACA 3′) was used as probe after labeling using [γ^{32}P] ATP and T4 polynucleotide kinase (Invitrogen). As a loading control a DNA complementary to U6 snRNA (5′ CCAATTTTATCGGATGTCCCCG 3′) was used as probe. Hybridizations were performed at 42°C for 19 h in Rapid-hyb buffer (GE Healthcare). Hybridized membranes were washed twice in 2x SSC/0.1% SDS for 45 min each time and then exposed to the Phosphor Screen System (GE Healthcare). Each miRNA blot was repeated three times, representative results are shown. The intensity of each hybridization band was quantified by densitometry using the ImageQuant 5.2 software (Molecular Dynamics).

Real-time quantitative RT-PCR (qRT-PCR)

To quantify transcript levels of mature miRNAs cDNA was synthesized from 1 µg total RNA using NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen) or RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) for transcripts of target genes. Resulting cDNAs were then diluted 10-fold and used to perform the qRT-PCR experiments using SYBR Green qPCR Master Mix (Fermentas) following manufacturer's instructions. Briefly, each reaction (15 µl) contained 7.5 µl of SYBR Green, 100 nM forward primer, 100 nM universal primer and 2 µl diluted cDNA. DNase/RNase-free water was used to adjust the volume to 15 µl. The reaction mix was then incubated in a 96 well plate and analyzed using iQ5 Real-Time PCR Detection System and iQ5 Optical System Software (Bio-Rad). The thermal cycler settings were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 55°C for 20 s. This cycle was followed by a melting curve analysis ranging from 50 to 95°C, with temperature increasing steps of 0.5°C every 10 s. Melting curves for each amplicon were observed carefully to confirm the specificity of the primers used. All qRT-PCR reactions were made by duplicate in iCycler BioRad equipment and at least two independent experiments were performed. Relative transcript levels for each sample were obtained using the 'comparative C_t method'. The threshold cycle (C_t) value obtained after each reaction was normalized to the C_t value of miR159 for miRNA levels or to the C_t value of the ubiquitin (UBC) or EF1α genes for gene levels. The expression of reference genes was constant across the conditions. The relative expression level was obtained by calibrating the $\Delta\Delta C_t$ values for the stressed conditions used and the normalized C_t value (ΔC_t) for the controls. Table S2 shows the sequences of the primers used for qRT-PCR amplification of *P. vulgaris* genes. Gene models for miR398b (Phvul.008G202400.1) and *CSD1* (Phvul.006G097000), experimentally validated as miR398 target in common bean, were identified by De la Rosa et al. (in preparation). *Nod19* as well as the common bean CuD responsive genes analyzed were identified after a BLAST search [32] in the common bean genome sequence (http://www.phytozome.net/search.php?method = Org_Athaliana) based in reported gene sequences from legumes. For *Nod19* the *M. truncatula* gene sequence (*MtN19*) was used for the BLAST analysis and Phvul.006G127400.1 was identified as the ortholog gene; this gene model is annotated as "Stress up-regulated Nod19". The common bean CuD-responsive genes identified and analyzed in this work were: *FSD* (Phvul.007G135400.1), *COPT* (Phvul.011G060400), *FRO* (Phvul.006G142300), *APX* (Phvul.011G071300). Though the *P. vulgaris* genome sequence (www.phytozome.net) gives more than one gene model for each analyzed gene, in each case we selected the one showing highest similarity with soybean orthologous genes considering that soybean has a well annotated genome sequence and it is phylogenetically close to common bean.

Plasmid construction

To obtain a miR398 over-expression construct, initially the pTDT-DC plasmid was constructed derived from the pTDT-DC-

RNAi vector [6]; it contained the 35S CaMV promoter, the attL gateway clonase reaction sites and the tdTomato gene (red-fluorescent protein, RFP) as a reporter gene. We did the construct to over-express miR398 prior to the release of the *P. vulgaris* genome sequence and so a clone from the *M. truncatula* miR398c precursor (MtrV 2Chr7_r3721) was used. Mature miRNA sequence of *M. truncatula* miR398c is identical to that of *P. vulgaris* miR398b. The *mtr-miR398c* precursor (358 bp) was cloned into the pENTR/SD/D-TOPO vector (Invitrogen) using specific forward (5′-CACCTCATTTCCATGACAACATGACA-3′) and reverse (5′-TTGTGCTTCCATCAACCAGT-3′) primers. LR clonase reaction (Gateway system, Invitrogen) between pTDT-DC and pENTR-precMiR398 provided the plasmid pOE398 to over-express miR398 under 35S promoter. To inhibit the activity of miR398c we proposed to use the artificial target mimicry strategy consisting in the expression of a modified sequence of *Pv4* (*IPS1*) containing an imperfect complementary sequence to miR398 that would reduce the miRNA-induced cleavage of its target genes [57]. The specific miR399-recognition site within *Pv4* (*IPS1*) (TC7206, Bean Gene Index DFCI) sequence was modified in vitro to obtain a mimicry sequence to miR398. We used an overlapping PCR strategy consisting in two PCR reactions: PCR1 [Pv4-Fwd (5′-CACCCAACACTCCTTCTCAAATCCTCTC-3′) + amiR 398-Rev 5′- *tgtgttctcaaactgtcgcccctt*TTCAAGAGAAAATCGCC-3′] and PCR2 [amiR398-Fwd (5′- *aaggggcgacagtttgagaacaca*TTTTCC-TATTCCTGGAACTCAC-3′) + Pv4-Rev 5′AGTAAGAAG-CAATTTTGTTTTG 3′], the products were later mixed to obtain the Pv4 modified complete sequence. The sequence obtained was introduced into pTDT-DC vector. The empty pTDT-DC vector (used as a control, hereafter termed EV) and the resulting OE398 and pMIM398 plasmids were introduced by electroporation into *Agrobacterium rhizogenes* K599, which was then used for plant transformation.

Fungal infection assay

Cultures of *Sclerotinia sclerotiorum* were started 48 h prior to inoculation by sub-culturing actively growing edges of fungal colonies from stock cultures onto potato dextrose agar (DIFCO). Inoculation of trifoliate leaves from young *Nicotiana benthamiana* or *P. vulgaris* plants was performed as described by Valdés-López et al. [78]. Briefly, leaves were detached and floated for 16 h in 20 ml of water in a Petri dish. Then, leaves were transferred into a Petri dish (one per trifolium) that contained moistened Whatman paper. One agar plug (4 mm diameter) with growing mycelium was placed on each leaf. Petri dishes were sealed with Parafilm and then placed in a growth chamber with controlled environmental conditions. One or two days after inoculation *P. vulgaris* or *N. benthamiana* leaves, respectively, were harvested and *S. sclerotiorum* infection levels were determined by measuring the lesion size. After this, leaves were frozen in liquid nitrogen and stored until used. Expression pattern of miR398b or target genes in fungal infected leaves was determined by qRT-PCR. The experiment was repeated three times, each at different dates and with new inoculum, to obtain three biological replicates.

Plant transformation

For common bean transformation the protocol described by Estrada-Navarrete et al. [56] with minor modifications [79] was used to obtain composite plants with transgenic roots. Plantlets were infected with the *Agrobacterium rhizogenes* K599 strain carrying previously described constructs (EV, OE398 or MIM398). Plant growth for hairy root formation and confirmation of the expression of the reporter gene in transgenic hairy roots were done as reported [79]. Composite common bean plants carrying only fluorescent hairy roots were transferred to a hydroponic system. After 7 days of growth adaptation in hydroponics, the composite plants were transferred to control or stress treatments as described above.

For transformation of *Nicotiana benthamiana* leaves, *Agrobacterium tumefaciens* LBA4404 strain was transformed with the respective binary constructs (EV, OE398) via electroporation and grown in Luria-Broth agar / spectinomycin (100 μg/ml) plates. Just prior to the plant infiltration, a small amount of bacteria were scrapped from the plate and dissolved in 10 mM $MgCl_2$. Each bacterial suspension was adjusted to $OD_{600} = 0.3$, and then incubated with 10 μM acetosyringone at room temperature for 2 h. Fully expanded *N. benthamiana* leaves were infiltrated by using needleless syringe. Plants were kept for three days in a growth chamber with 25°C temperature, 70% humidity and natural illumination. Leaves showing RFP fluorescence were harvested for *S. sclerotiorum* infection experiments.

Supporting Information

Table S1 Expression profile of miR398a.
(DOC)

Table S2 Primer sequences for qRT-PCR.
(XLS)

Acknowledgments

We are grateful to Alfonso Leija, Sara I. Fuentes and Victor Bustos for technical assistance. We acknowledge Dr. Manuel Becana (CSIC, Spain) for his interest in miR398 role in oxidative stress of legumes that encouraged this work.

Author Contributions

Conceived and designed the experiments: LN SP OVL JLR GH. Performed the experiments: LN SP OVL ABMS BNF GSV. Analyzed the data: LN SP OVL ABMS JLR GH. Contributed reagents/materials/analysis tools: LN SP OVL ABMS JLR GH. Wrote the paper: LN SP OVL GH.

References

1. Rogers K, Chen X (2013) Biogenesis, turnover and mode of action of plant microRNAs. Plant Cell 25: 2383–2399.
2. Voinnet O (2009) Origin, biogenesis and activity of plant microRNAs. Cell 136: 669–687.
3. Broughton WJ, Hernández G, Blair M, Beebe S, Gepts P et al. (2003) Beans (*Phaseolus spp.*) – model food legumes. Plant and Soil 252: 55–128.
4. Arenas-Huertero C, Pérez B, Rabanal F, Blanco-Melo D, De la Rosa C et al. (2009) Conserved and novel miRNAs in the legume *Phaseolus vulgaris* in response to stress. Plant Mol Biol 70: 385–401.
5. Valdés-López O, Yang SS, Aparicio-Fabre R, Graham PH, Reyes JL et al. (2010) MicroRNA expression profile in common bean (*Phaseolus vulgaris*) under nutrient deficiency stresses and manganese toxicity. New Phytol 187: 805–818.
6. Valdés-López O, Arenas-Huertero C, Ramírez M, Girard L, Sánchez F et al. (2008) Essential role of MYB transcription factor: PvPHR1 and microRNA: PvmiR399 in phosphorus-deficiency signalling in common bean roots. Plant Cell Environ 31: 1834–1843.
7. Peláez P, Trejo MS, Íñiguez LP, Estrada-Navarrete G, Covarrubias AA et al. (2012) Identification and characterization of microRNAs in *Phaseolus vulgaris* by high-throughput sequencing. BMC Genomics 13: 83. Available: http://www.biomedcentral.com/1471-2164/13/83. Accessed 6 March 2012.
8. Lelandais-Brière C, Naya L, Sallet E, Calenge F, Frugier F et al. (2009) Genome-wide *Medicago truncatula* small RNA analysis revealed novel microRNAs and isoforms differentially regulated in roots and nodules. Plant Cell 21: 2780–2796.

9. De Luis A, Markmann K, Cognat V, Holt DB, Charpentier M, Parniske M et al. (2012) Two microRNAs linked to nodule infection and nitrogen-fixing ability in the legume *Lotus japonicus*. Plant Physiol 160: 2137–2154.
10. Song QX, Liu YF, Hu XY, Zhang WK, Ma B et al. (2011) Identification of miRNAs and their target genes in developing soybean seeds by deep sequencing. BMC Plant Biol 11: 5, . Available: http://www.biomedcentral.com/1471-2229/11/5. Accessed 10 January 2011.
11. Zhao CZ, Xia H, Frazier TP, Yao YY, Bi YP et al. (2010) Deep sequencing identifies novel and conserved microRNAs in peanuts (*Arachis hypogaea* L.). BMC Plant Biol 10: 3, . Available: http://www.biomedcentral.com/1471-2229/10/3. Accessed 5 January 2010.
12. Paul S, Kundu A, Pal A (2013) Identification and expression profiling of *Vigna mungo* microRNAs from leaf small RNA transcriptome by deep sequencing. J Integr Plant Biol doi: 10.1111/jipb.12115
13. Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell 14: 787–799.
14. Bonnet E, Wuyts J, Rouze P, Van de Peer Y (2004) Detection of 91 potential conserved plant microRNAs in *Arabidopsis thaliana* and *Oryza sativa* identifies important target genes. Proc Natl Acad Sci U S A 101: 11511–11516.
15. Beauclair L, Yu A, Bouche N (2010) microRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNA in Arabidopsis. Plant J 62: 454–462.
16. Sunkar R, Kapoor A, Zhu JK (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell 18: 2051–2065.
17. Jia X, Wang WX, Ren L, Chen QJ, Mendu V et al. (2009) Differential and dynamic regulation of miR398 in response to ABA and salt stress in *Populus tremula* and *Arabidopsis thaliana*. Plant Mol Biol 71: 51–59.
18. Siré C, Moreno AB, Garcia-Chapa M, Lopez-Moya JJ, San SB (2009) Diurnal oscillation in the accumulation of *Arabidopsis* microRNAs, miR167, miR168, miR171 and miR398. FEBS Lett 583: 1039–1044.
19. Zhou ZS, Wang SJ, Yang ZM (2008) Biological detection and analysis of mercury toxicity to alfalfa (*Medicago sativa*) plants. Chemosphere 70: 1500–1509.
20. Zhou ZS, Zeng HQ, Liu ZP, Yang ZM (2012) Genome-wide identification of *Medicago truncatula* microRNAs and their targets reveals their differential regulation by heavy metal. Plant Cell Environ 35: 86–99.
21. Liang G, He H, Yu D (2012) Identification of nitrogen starvation-responsive microRNAs in *Arabidopsis thaliana*. PLoS One 7(11): e48951. doi:10.1371/journal.pone.0048951
22. Guan Q, Lu X, Zeng H, Zhang Y, Zhu J (2013) Heat stress induction of miR398 triggers a regulatory loop that is critical for thermotolerance in Arabidopsis. Plant J 74: 840–851.
23. Trindade I, Capitao C, Dalmay T, Fevereiro MP, Santos DM (2010) miR398 and miR408 are up-regulated in response to water deficit in *Medicago truncatula*. Planta 231: 705–716.
24. Hsieh JC, Lin SI, Shih AC, Chen JW, Lin WY et al. (2009) Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. Plant Physiol 151: 2120–2132.
25. Kuo HF, Chiou TJ (2011) The role of microRNAs in phosphorus deficiency signaling. Plant Physiol 156: 1016–1024.
26. Yamasaki H, Abdel-Ghany SE, Cohu CM, Kobayashi Y et al. (2007) Regulation of copper homeostasis by micro-RNA in *Arabidopsis*. J Biol Chem 282: 16369–16378.
27. Yamasaki H, Hayashi M, Fukazawa M, Kobayashi Y, Shikanai T (2009) SQUAMOSA promoter binding protein-like7 is a central regulator for copper homeostasis in *Arabidopsis*. Plant Cell 21: 347–361.
28. Dugas DV, Bartel B (2008) Sucrose induction of *Arabidopsis* miR398 represses two Cu/Zn superoxide dismutases. Plant Mol Biol 67: 403–417.
29. Jagadeeswaran G, Saini A, Sunkar R (2009) Biotic and abiotic stress down-regulate miR398 expression in Arabidopsis. Planta 229: 1009–1014.
30. Sunkar R, Zhu JK (2004) Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. Plant Cell 16: 2001–2019.
31. Gamas P, Niebel FC, Lescure N, Cullimore J (1996) Use of a subtractive hybridization approach to identify new *Medicago truncatula* genes induced during root nodule development. Mol Plant Microbe Interact 9: 233–242.
32. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–402.
33. Moreau S, Verdenaud M, Ott T, Letort S, de Billy F et al. (2011) Transcription reprogramming during root nodule development in *Medicago truncatula*. PLoS One 6(1): e16463. doi:10.1371/journal.pone.0016463
34. Vernié T, Moreau S, de Billy F, Plet J, Combier JP et al. (2008) EFD Is an ERF transcription factor involved in the control of nodule number and differentiation in *Medicago truncatula*. Plant Cell 20: 2696–2713.
35. Doss RP (2005) Treatment of pea pods with Bruchin B results in up-regulation of a gene similar to MtN19. Plant Physiol Biochem 43: 225–231.
36. Kimura M, Yamamoto YY, Seki M, Sakurai T, Sato M et al. (2003) Identification of *Arabidopsis* genes regulated by high light-stress using cDNA microarray. Photochem Photobiol 77: 226–233.
37. Ganesan V, Thomas G (2001) Salicylic acid response in rice: influence of salicylic acid on H_2O_2 accumulation and oxidative stress. Plant Sci 160: 1095–1106.
38. Li J, Brader G, Palva ET (2004) The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell 16: 319–331.
39. Park HC, Kim ML, Kang YH, Jeon JM, Yoo JH et al. (2004) Pathogen- and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. Plant Physiol 135: 2150–2161.
40. Yanhui C, Xiaoyuan Y, Kun H, Meihua L, Jigang L et al. (2006) The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol Biol 60: 107–124.
41. Puig S, Andrés-Colas N, García-Molina A, Peñarrubia L (2007) Copper and iron homeostasis in *Arabidopsis*: responses to metal deficiencies, interactions and biotechnological applications. Plant Cell Environ 30: 271–290.
42. Fernandes JC, Henriques FS (1991) Biochemical, physiological and structural effects of excess copper in plants. Bot Rev. 57: 246–273.
43. Märschner H (2002) Mineral nutrition in higher plants. 3rd edition. Academic Press, London UK.
44. El-Nennah M, El-Kobbia T, Shehata A, El-Gamal I (1982) Effect of irrigation of loamy sand soil by sewage effluents on its content of some nutrients and heavy metals. Plant Soil 65: 289–292.
45. Oldenkamp L, Smilde KW (1966) Copper deficiency in Douglas fir (Pseudotsuga menziesii (Mirb.) Franco). Plant Soil 25: 150–152.
46. Cuypers A, Koistinen K, Kokko H, Kärenlampi S, Auriola S et al. (2005) Analysis of bean (*Phaseolus vulgaris* L.) proteins affected by copper stress. J Plant Physiol 162: 383–392.
47. Bouazizi H, Jouili H, Geitmann A, El Ferjani E (2010) Copper toxicity in expanding leaves of *Phaseolus vulgaris* L.: antioxidant enzyme response and nutrient element uptake. Ecotox Environ Safe 73: 1304–1308.
48. Cuypers A, Vangronsveld J, Clijsters H (2000) Biphasic effect of copper on the ascorbate-glutathione pathway in primary leaves of *Phaseolus vulgaris* seedlings during the early stages of metal assimilation. Physiol Plantarum 110: 512–517.
49. Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373–399.
50. Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48: 251–275.
51. Schützendübel A, Polle A (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J Exp Bot 53: 1351–1365.
52. Sgherri C, Quartacci MF, Navari-Izzo F (2007) Early production of activated oxygen species in root apoplast of wheat following copper excess. J Plant Physiol 164: 1152–1160.
53. Sharma SS, Dietz KJ (2009) The relationship between metal toxicity and cellular redox imbalance. Trends Plant Sci 14: 43–50.
54. Abdel-Ghany SE, Pilon M (2008) MicroRNA-mediated systemic down-regulation of copper protein expression in response to low copper availability in *Arabidopsis*. J Biol Chem 283: 15932–15945.
55. Li Y, Zhang QQ, Zhang J, Wu L, Qi Y, et al. (2010) Identification of microRNAs involved in pathogen associated molecular pattern-triggered plant innate immunity. Plant Physiol 152: 2222–2231.
56. Estrada-Navarrete G, Alvarado-Affantranger X, Olivares JE, Guillén G, Díaz-Camino C et al. (2007) Fast, efficient and reproducible genetic transformation of *Phaseolus spp*. by *Agrobacterium rhizogenes*. Nat Protoc 2: 1819–1824.
57. Franco-Zorrilla JM, Valli A, Todesco M, Mateos I, Puga MI et al. (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. Nat Genet 39: 1033–1037.
58. Winkel-Shirley B (2002) Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5: 218–223.
59. Bongue-Bartelsman M, Phillips DA (1995) Niutrogen stress gene expression of enzymes in the flavomoid biosynthestic pathway of tomato. Plant Physiol Biochem 33: 539–546.
60. Perea-García A, García-Molina A, Andres-Colas N, Vera-Sirera F, Pérez-Amador MA et al. (2013) Arabidopsis copper transport protein COPT2 participates in the cross talk between iron deficiency responses and low-phosphate signaling. Plant Physiol 162: 180–194.
61. Robinson NJ, Procter CM, Connolly EL, Guerinot ML (1999) A ferric-chelate reductase for iron uptake from soils. Nature 397: 694–697.
62. Mukherjee I, Campbell NH, Ash JS, Connolly EL (2006) Expression profiling of the Arabidopsis ferric chelate reductase (FRO) gene family reveals differential regulation by iron and copper. Planta 223: 1178–1190.
63. Baron C, Zambryski PC (1995) The plant response in pathogenesis, symbiosis, and wounding: variations on a common theme? Annu Rev Genet 29: 107–129.
64. Oldroyd G (2013) Speak, friend and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbiol 11: 252–263.
65. Cárdenas L, Martínez A, Sánchez F, Quinto C (2008) Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs). Plant J 56: 802–813.
66. Mandon K, Pauly N, Boscari A, Brouquisse R, Frendo P et al. (2009) ROS in the Legume-*Rhizobium* Symbiosis. In: Rio LA, Puppo A, editors. Reactive Oxygen Species in Plant Signaling. Berlin, Heidelberg: Springer-Verlag. pp.135–147.
67. Santos R, Herouart D, Sigaud S, Touati D, Puppo A (2001) Oxidative burst in alfalfa-*Sinorhizobium meliloti* symbiotic interaction. Mol Plant Microbe Interact 14: 86–89.

68. Ramu SK, Peng HM, Cook DR (2002) Nod factor induction of reactive oxygen species production is correlated with expression of the early nodulin gene rip1 in *Medicago truncatula*. Mol Plant Microbe Interact 15: 522–528.
69. Zhang W, Gao S, Zhou X, Chellappan P, Chen Z et al. (2011) Bacteria responsive microRNAs regulate plant innate immunity by modulating plant hormone networks. Plant Mol Biol 75: 93–105.
70. Purdy LH (1979) *Sclerotinia sclerotiorum*: history, disease and symptomatology, host range, geographic distribution and impact. Phytopathology 69: 875–880.
71. Kim HJ, Chen C, Kabbage M and Dickman MB (2011) Identification and characterization of *Sclerotinia sclerotiorum* NADPH oxidase. Appl Environ Microbiol 77: 7721–7729.
72. Zhou J, Sun A and Xing D (2013) Modulation of cellular redox status by thiamine-activated NADPH oxidase confers *Arabidopsis* resistance to *Sclerotinia sclerotiorum*. J Exp Bot 64: 3261–3272.
73. Williams B, Kabbage M, Kim HJ, Britt R and Dickman MB (2011) Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. PLoS Pathog 7(&): e1002107. doi:10.1371/journal.ppat.1002107
74. Franco AA, Munns DN (1982) Nodulation and growth of *Phaseolus vulgaris* in solution culture. Plant Soil 66: 149–160.
75. Giusti MM, Wrolstad RE (2001) Characterization and measurement of anthocyanins by UV-visible spectroscopy. In: Wrolstad RE, Acree TE, Decker EA, Penner MH, Reid DS, Schwartz SJ, Shoemaker CF, Smith DM, Sporns P, editors. Current Protocols in Food Analytical Chemistry. New Jersey, John Wiley and Sons, Inc. F1.2.1–F1.2.13
76. Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD et al. (2012) Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res 40: D1178-D1186.
77. Chang WC, Lee TY, Huang HD, Huang HY, Pan RL (2008) PlantPAN: Plant promoter analysis navigator, for identifying combinatorial cis-regulatory elements with distance constraint in plant gene groups. BMC Genomics 9: 561, . Available: http://www.biomedcentral.com/1471-2164/9/561. Accessed 26 November 2008.
78. Valdés López O, Thibivilliers S, Qiu J, Wezxhing W, Nguyen THN et al. (2011) Identification of quantitative trait loci controlling gene expression during innate immunity response of soybean. Plant Physiol 157: 1975–1986
79. Aparicio-Fabre R, Guillén G, Loredo M, Arellano J, Valdés-López O et al. (2013) Common bean (*Phaseolus vulgaris* L.) PvTIFY orchestrates global changes in transcript profile response to jasmonate and phosphorus deficiency. BMC Plant Biol 13: 26, . Available: http://www.biomedcentral.com/1471-2229/13/26. Accessed 13 February 2013