UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DOCTORADO EN CIENCIAS BIOMÉDICAS

FACULTAD DE ESTUDIOS SUPERIORES IZTACALA

Determinación del papel de Argonauta5 en el establecimiento de la simbiosis leguminosa-rizobio.

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

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INDICE

RESUMEN

La simbiosis entre leguminosas y rizobios ha sido ampliamente estudiada para conocer los diferentes componentes que la regulan. Dentro de estos, los microRNAs (miRNAs) han sido evaluados tanto en etapas tempranas como en eventos tardíos. Sin embargo, el papel en esta simbiosis de las proteínas Argonauta (AGO), las cuales son cruciales para el funcionamiento de los miRNAs, no ha sido estudiado. Junto con la proteína AGO, se forma el complejo de silenciamiento inducido por RNA (RISC, por sus siglas en inglés) el cual media la función de los miRNAS. La proteína AGO5 está involucrada en procesos de defensa en plantas de *Arabidopsis thaliana* infectadas con el virus de la papa, lo cual demuestra su papel en la interacción planta-patógeno. A pesar de esta evidencia, el papel de AGO5 en la regulación de interacciones simbióticas no había sido abordado. En el presente trabajo se analizó el papel de AGO5 en el establecimiento de la simbiosis leguminosa-rizobio, teniendo como modelo a las interacciones simbióticas *Glycine max-Bradyrhizobium diazoefficiens* y *Phaseolus vulgaris-Rhizobium tropici*. Nuestros resultados muestran que la expresión de *AGO5* se induce desde la primera hora en que la leguminosa tiene contacto con el rizobio. Además, a través de la actividad del promotor de *AGO5* fusionado a GUS, se observó que *AGO5* se expresa en pelos radiculares deformados por rizobios y en meristemos nodulares. El silenciamiento mediante RNA de interferencia de *AGO5* confirmó su papel en el establecimiento de la simbiosis leguminosa-rizobio, ya que este derivó en una disminución en el número de pelos radicales deformados por rizobio y en el número y tamaño de nódulo. En conjunto, los datos reportados en este estudio informan que el silenciamiento de AGO5 afecta los procesos de infección y desarrollo de nódulo, indicando que esta proteína AGO y sus RNAs pequeños asociados son cruciales para el establecimiento exitoso de la simbiosis leguminosa-rizobio.

ABSTRACT

The symbiosis between legumes and rhizobia has been extensively studied to know the different components that regulate it. MicroRNAs (miRNAs) have been evaluated in both early and advanced stages of the symbiosis. However, the role of Argonaute proteins (AGO), which are crucial for the function of miRNAs has not been studied. Together with the ARGONAUTE protein, the RNA-induced silencing complex (RISC) is formed which mediates the function of miRNAs. Argonauta 5 (AGO5) protein is involved in defense processes in *Arabidopsis thaliana* plants infected with the potato virus. This indicates its role in plant-pathogen interaction. However, its role in symbiosis has not been studied. In this work, the role of AGO5 in the establishment of the legume-rhizobia symbiosis was analyzed, in the model for symbiotic interactions *Glycine max-Bradyrhizobium diazoefficiens* and *Phaseolus vulgaris-Rhizobium tropici.* Our results show that the expression of *AGO5* was induced from the first hour in which the legume has contact with the rhizobia. Furthermore, through the activity of the AGO5 promoter fused to GUS, AGO5 was observed to be expressed in deformed root hairs and nodular meristem. Silencing of *AGO5* by RNA interference confirmed its role in legume-rhizobia symbiosis establishment since it resulted in a decrease in the number of deformed root hairs by rhizobia and the number and size of the nodule. Altogether, the data reported in this study evidenced that the silencing of AGO5 affects the processes of infection and nodule development, indicating that AGO protein and its small RNAs associated is crucial for the successful establishment of the legume-rhizobia symbiosis.

1. INTRODUCCION

1.1 El nitrógeno: importancia y problemáticas.

El nitrógeno (N) es uno de los nutrimentos más importantes requerido por las plantas para completar su ciclo de vida y desarrollo. La importancia del N radica en que este elemento es un componente esencial de la mayoria de las biomoléculas, incluyendo los aminoácidos, proteínas y ácidos nucleicos (Ferguson, *et al.,* 2010). El N se encuentra en una alta concentración en su forma gaseosa (N_2), el cuál no puede ser utilizado directamente por las plantas, ya que ellas solamente incorporan a su metabolismo formas asimilables (i.e., nitrato, amoníaco o aminoácidos), las cuales están en bajas concentraciones en el suelo. Por lo anterior, la baja disponibilidad de N asimilable es uno de los principales factores que limitan el crecimiento, desarrollo y producción de plantas de interés agronómico a nivel mundial (Ferguson, *et al.,* 2010). La agricultura mundial por varios años ha optado por el uso de fertilizantes sintéticos como la principal estrategia para disminuir los efectos de la deficiencia de N y asegurar la producción de semillas de alta calidad para consumo humano (Gutiérrez, 2012; Delaux, *et al.,* 2015).

El uso de ferilizantes sintéticos de N, además de los benificios innegables en la seguridad alimenticia, también ha ocasionado daños irreversibles al ambiente. Por ejemplo, para su producción mediante el proceso Haber-Bosh se requiere de la combustión de grandes cantidades de combustible fósil (i.e., gas natural y petróleo), lo que conlleva a la liberación de gases de efecto invernadero (i.e., CO_2 y N_2O) que contribuyen al calentamiento global (Galloway, *et al*., 2004; Crutzen, *et al.,* 2007). Aunado a esto, el precio de los combustibles fósiles va incrementando y por ende el precio de los fertilizantes sintéticos (Crutzen, *et al*., 2007). Otro problema relacionado con los fertilizantes sintéticos es su uso desmedido. Por lo regular, los agricultores agregan cantidades de fertilizantes superiores a las que las plantas pueden y necesitan absorber, el resto del fertilizante se lixivia a la solución del suelo, provocando su salinización y la eutrofización de cuerpos de agua (Crutzen, *et al.,*

2007; Rockstrom, *et al.,* 2009). Aproximadamente un tercio de los cultivos utilizados para consumo humano depende de los fertilizantes sintéticos nitrogenados (Smil, 1997), por lo que su síntesis y aplicación conlleva a un incremento en el deterioro ambiental que incluye la pérdida de suelos arables, eutroficación de cuerpos de agua, e incremento de la emisión de gases de efecto de invernadero.

Una de las prácticas más antiguas para la aportación de N en los suelos es el cultivo de las leguminosas (Dalby, 1998). Las leguminosas, sólo después de los cereales, son el segundo cultivo más importante en la seguridad alimenticia ya que son una excelente fuente de proteínas y minerales para consumo humano (Bazin, *et al*., 2012; Castro-Guerrero *et al*., 2016). Las leguminosas también poseen una gran relevancia ecológica, dada su capacidad de establecer asociaciones simbióticas con bacterias fijadoras de N denominadas rizobios (Foyer, *et al*., 2016). A través de esta simbiosis, las leguminosas no solamente obtienen N asimilable para satisfacer sus necesidades metabólicas, si no que también enriquece el suelo con fuentes asimilables de N (Roy, *et al*., 2019). Se ha estimado que a través de la fijación simbiótica de nitrógeno (FSN) se incorporan aproximadamente 50 millones de toneladas de N asimilable por año, lo que beneficia a otras plantas no leguminosas (Canfield *et al*, 2010; Roy, *et al*., 2019). Aunque no es comparable con lo que se utiliza en fertilizantes químicos, el N_2 es convertido a formas asimilables para las plantas, sin las problemáticas económicas y ambientales de los fertilizantes sintéticos (Canfield *et al*, 2010, Oldroy & Dixon, 2014; Bakken & Frostegård, 2017; Roy, *et al.,* 2019). Por lo que el estudio de la FSN es una alternativa ante la creciente problemática del N, que implica la producción de alimento que garantice la seguridad alimentaria (Oldroyd & Dixon, 2014; Bakken & Frostegård, 2017).

1.2 La FSN

Dentro de las bacterias fijadoras de nitrógeno, existe la familia *Rhizobiaceae*, que incluye a los generos *Rhizobium, Bradyrhizobium, Sinorhizobium* y *Azorhizobium,* los cuales son llamados de manera colectiva como “rizobios” (Hirsch, 1992). Los rizobios establecen interacciones simbióticas con plantas leguminosas (con excepción de la no leguminosa *Parasponia*) y son capaces de reducir el N_2 en NH_4 gracias a que poseen una enzima nitrogensa (Udvardi & Day, 1997; Oldroyd & Downie, 2004). De tal forma que las leguminosas son colonizadas por los rizobios a través de los pelos radicales y estos son albergados en órganos especializados denominados nódulos (Ferguson *et al*., 2010; Oldroyd & Dixon, 2014). En el nódulo, el rizobio se convierte en un bacteroide que en conjunto con una membrana de origen vegetal forma un simbiosoma. En el simbiosoma se lleva a cabo la fijación de N_2 en un ambiente con bajos niveles de oxígeno, pero suficiente para energetizar el proceso de fijación de nitrógeno, el cual requiere al menos 16 móleculas de ATP por cada mólecula de N_2 reducido (Vazquez-Limon, 2012). Para sostener esta simbiosis, la leguminosa debe proveer niveles bajos de oxígeno al rizobio a través de la producción de hemoglobinas vegetales denominadas leghemoglobinas. Asimismo, la leguminosa debe aportar grandes cantidades de fuentes de carbono al bacteroide para garantizar la cantidad precisa de ATP. A cambio, la leguminosa recibe fuentes de N asimilable que pueden ser NH_4 o diferentes tipos de aminoácidos (Lodwig, *et al*., 2003; Oldroyd & Downie, 2004; Ferguson *et al.,* 2010; Oldroyd & Dixon, 2014; Roy, *et al*., 2019).

La simbiosis leguminosa-rizobio se establece mediante dos procesos altamente coordinados: la infección bacteriana y la organogénesis del nódulo (Oldroyd & Downie, 2008; Ferguson, *et al*., 2010). Ambos procesos son regulados por al menos dos programas genéticos, los cuales son finamente coordinados en espacio y tiempo, esto con la finalidad de que la infección por el rizobio llegue al nódulo en formación y culmine con la invasión y colonización de las células de este nuevo

órgano (Madsen, *et al*., 2010). Para activar estos programas genéticos se requiere de un diálogo molecular entre la leguminosa y el rizobio, en el cual el reconocimiento de moléculas señal liberadas por el rizobio activa la cascada de señalización en la planta (Oldroyd & Downie, 2008) que le permite controlar el acceso del rizobio y la formación de nódulos (Figura 1) (Genre, *et al*., 2005; Genre & Bonfante, 2007; Oldroyd & Downie, 2008; Oldroyd, *et al*., 2011).

1.2.1 Diálogo molecular entre leguminosas y rizobios: Liberación de la molécula señal.

La simbiosis leguminosa-rizobio inicia cuando los niveles de N en la planta y en la rizosfera son bajos. Ante esta situación, la leguminosa excreta flavonoides e isoflavonoides hacia la rizosfera, cuya naturaleza química es dependiente de la especie de leguminosa que los excreta. Estas moléculas señal funcionan como quimioatrayentes para los rizobios compatibles hacia los pelos radiculares de la leguminosa (Liu & Murray, 2016; Roy *et al*., 2019). Además, la detección de los (iso)/flavonoides activa la transcripción de los genes *nod* en los rizobios, los cuales están involucrados en la biosíntesis de moléculas de lipoquitooligosacáridos (LCOs) con decoraciones químicas específicas en cada especie de rizobio denominados Factores de Nodulación (FNs) (Debrosses, & Stougaard, 2011; Liu & Murray, 2016). Los LCOs son la estructura general de FNs, la cual varía entre especies de rizobios. Las variaciones en los LCOs radican en el número de residuos de glucosamina, longitud y saturación de la cadena alifática (Denarie, *et al.*, 1996; Downie, 1998;) y modificaciones químicas adicionales que incluyen la adición de grupos metilo, sulfurilo, acetilo entre otros (Oldroyd & Downie, 2004). Tanto la naturaleza química de los (iso)/flavonoides y de los FNs hacen que la simbiosis entre leguminosas y rizobios sea altamente específica, lo que implica que cada especie de leguminosa interaccione con un grupo específico de rizobios y viceversa (Perret, *et al.,* 2000; Oldroyd & Downie, 2008; Oldroyd, 2013; Liu & Murray, 2016; Roy *et al*., 2019). Además de los FNs, existen otras moléculas que ayudan a conservar la

especificidad durante el diálogo molecular, las cuales son principalmente exopolisacáridos (EPS) que están en la superficie de la bacteria. Los EPS sirven para aportar especificidad a la simbiosis, principalmente durante el proceso de infección, especialmene en la elongación del hilo de infección (Laus, *et al*., 2005; Kawaharada, *et al*., 2015).

1.2.2 Diálogo molecular entre leguminosas y rizobios: Percepción de la señal simbiótica

Estudios genéticos y bioquímicos en las leguminosas modelo *Medicago truncatula* y *Lotus japonicus* han llevado a la identificación de receptores de FNs y de EPS (Madsen, *et al*., 2003; Kawaharada, *et al*., 2015). Por ejemplo, en los pelos radicales, donde ocurre el reconocimiento mutuo entre ambos simbiontes, se ha demostrado la existencia de dos receptores tipo cinasa LysM encargados de detectar los FNs, llamados Nod Factor Receptor1 (NFR1) y NFR5 en *L. japonicus*, cuyos ortólogos en *M. truncatula* son LYK3 y Nod Factor Perception (NFP), respectivamente (Limpens, *et al*., 2003; Madsen, *et al*., 2003; Radutoui, *et al*., 2003; Arrighi, *et al*., 2006; Indrasumunar 2007; Indrasumunuar, *et al*., 2009; Op den Camp, *et al*., 2011). Estos receptores constan de tres dominios, uno intracelular, uno transmembranal y otro extracelular con motivos LysM (Ferguson, *et al*., 2010). El dominio cinasa de NFR1 interactúa con NFR5, cuyo dominio cinasa carece de actividad cinasa (Limpens, *et al*., 2003; Madsen, *et al*., 2003; Radutoiu, *et al*., 2003; Indrasumunar, 2007; Indrasumunar, *et al*., 2009) para formar un heteródimero capaz de transmitir la señal de los FNs río abajo y desencadenando cascadas de señalización necesarias para la simbiosis (Limpens, *et al*., 2003; Madsen, *et al*., 2003; Radutoiu, *et al*., 2003). La participación de estos receptores en el reconocimiento de los FNs fue demostrada en el análisis de la respuesta simbiótica en plantas mutantes de *L. japonicus.* Los pelos radicales de la plantas de *L. japonicus,* al estar en contacto con su simbionte (*Mesorhizobium loti*) se deforman al reconocer a la bacteria. En las plantas mutantes *nfr1* y *nfr5,* no existe deformación

de pelos radicales después de 24 horas de ser inoculadas con su simbionte o con FNs purificados, lo cual demostró que estos dos receptores participan en el reconocimiento de los FNs (Radutoiu, *et al*., 2003).

Recientemente se ha demostrado la participación de otros receptores en el reconocimiento de los FNs, por ejemplo, en la epidermis de raíces de *L. japonicus* existe un tercer receptor de FNs, se trata de un receptor tipo LysM con un dominio cinasa intracelular, el cual por encontrarse en la epidermis fue nombrado NFRe (epidermal LysM receptor) (Murakami, *et al.,* 2018). El receptor NFRe reconoce los FNs y posteriormente fosforila a NFR5 para que se active la cascada de señalización que involucra la formación del nódulo. En las plantas mutantes *nfre* se observó que la mutación en este receptor afecta la nodulación (50% menos nódulos) sin que afecte el proceso de infección (Murakami, *et al.,* 2018). *La* expresión de NFRe suele ser baja pero constante y se expande por el sistema radicular (Murakami, *et al*., 2018). Otro receptor que también participa interaccionando con NFR5 es el receptor llamado NiCK4 (NFR5-interacting cytoplasmic kinase 4) (Wong, *et al.,* 2019). NiCK4 se localiza en la membrana plásmatica y esta relacionado con la percepción de los FNs. Mediante ensayos *in vivo* se estableció que se asocia con NFR5 fosforilando su dominio citoplásmico, contribuyendo a la señalización requerida para la nodulación, lo cual fue corroborado en plantas mutantes *nick4* que desarrollaban pocos nódulos (Wong, *et al*., 2019). La función de NiCK4 se encuentra río abajo de NFR5, debido a que no se detecta la señal inducida por NiCK4 en mutantes *nfr5*, lo que demostró que NFR5, junto con NFR1, son, si no los únicos, si los principales receptores de FNs (Murakami, *et al*., 2018; Wong, *et al*., 2019).

Adicional a los receptores de los FNs, también se ha demostrado la existencia de al menos un receptor de exopolisacáridos, el cual es denominado EPR3 (EXOPOLYSACCHARIDE RECEPTOR) y LYK10 en *L. japonicus y M. truncatula,* respectivamente (Kawaharada, *et al*., 2015; Maillet, *et al*., 2020). Estudios en ambas leguminosas modelo han demostrado que este receptor es específico para los EPS sintetizados por el rizobio y que estos receptores se activan después de la detección de los FNs e intervienen en el mecanismo de infección que involucra al hilo de infección, haciendo que este llegue hasta el primordio nodular. Las plantas mutantes

en estos receptores (*Ljepr3, Mtlyk10*) no forman hilos de infección elongados o no se puede sostener la infección desde la punta del pelo radical hasta la base del pelo y por ende la infección no llega a las células corticales (Kawaharada, *et al*., 2015; Kawaharada, *et al*., 2017; Maillet, *et al*., 2020).

Otro receptor tipo cinasa con repetidos de leucina (LRR) que interviene en la decodificación de la señal emitida por los FNs es el "Symbiosis Receptor-like Kinase (LjSYMRK) en *L. japonicus* o "Does not Make Infections2" (DMI2) en *M. truncatula* (Endre, *et al*., 2002, Stracke, *et al*., 2002; Mitra, *et al*., 2004; Capoen, *et al*., 2005; Indrasumunar, 2007). Este receptor se encuentra en la membrana plásmatica (Limpens, *et al*., 2005) y participa en la transducción de la señal requerida para los eventos tempranos que ocurren en el pelo radical (Endre, *et al*., 2002; Stracke, *et al*., 2002). Con este receptor se asocia la 3-hidroxi-3metilglutaril CoA reductasa (HMGR) que induce la síntesis de mevalonato, el cual sirve como mensajero secundario que permite la continuación de la cascada de señalización río abajo de los receptores de los FNs (Kevei, *et al*., 2007; Oldroyd, 2013).

1.2.3 Cascada de señalización: eventos moleculares.

El reconocimiento de los FNs implica la decodificación de una señal que dispare una cadena de señalización que involucra diferentes eventos moleculares, y que a su vez coordine los dos programas que actuarán a nivel del pelo radical y cortex de raíz para que la simbiosis se establezca (Ané, *et al*., 2004; Imaizumi-Ankaru, *et al*., 2005; Riely, *et al*., 2007; Ferguson, *et al*., 2010).

Una vez activados los receptores cinasa por los FNs, los canales iónicos, CASTOR y POLLUX en *L. japonicus* o DMI1 en *M. truncatula*, así como las nucleoporinas Nup133 y Nup85 son activados en la membrana nuclear. Estos canales iónicos y nucleoporinas permiten una liberación rápida y continua de calcio nuclear, las cuales son descritas como oscilaciones de calcio nuclear o "spiking" de calcio (Ané *et al*., 2004; Imaizumi-Ankaru, *et al*., 2005; Kanamori, *et al*., 2006; Riely, *et al*., 2007; Saito, *et al*., 2007; Sieberer, *et al*., 2009; Capoen, *et al*., 2011). Existen

evidencias de que el mensajero secundario mevalonato puede jugar un papel en la activación de los spiking de calcio; sin embargo, hasta el momento no se ha dilusidado el mecanismo preciso en el cual participe este mensajero secundario (Oldroyd, 2013). Otra respuesta activada por el reconocimiento de los FNs es la fosforilación de proteínas. En *M. truncatula* la proteína EPP1 (Early Phosphorylated Protein 1) se fosforila en respuesta al reconocimiento de los FNs (Valdés-López, *et al.,* 2019b, Anexo lll), participando en la cascada de señalización que induce el spiking de calcio. En plantas mutantes *Mtepp1*-RNAi se abolió casi por completo el spiking de calcio, lo que repercutió en la expresión de los genes simbióticos, ocasionando que estas plantas no desarrollaran nódulos (Valdés-López, *et al.,* 2019b, Anexo lll).

Los spiking de calcio son decodificados por una proteína nuclear dependiente de calcio y calmodulina denomidana CCaMK o DMI3 en *L. japonicu*s y *M. truncatula*, respectivamente (Levy, *et al*., 2004; Mitra, et a., 2004). La CCaMK/DMI3 fosforila al factor de transcripción CYCLOPS o IPD3 en *L. japonicus* y *M. truncatula*, respectivamente (Messinese, *et al*., 2007, Yano, *et al*., 2008; Oldroyd, *et al*., 2011). A su vez, CYCLOPS/IPD3 activa a los factores de transcripción Nodulation Signaling Pathway1 (NSP1; Smit, *et al*., 2005) y NSP2 (Kalo *et al*., 2005), los cuales forman un heterodímero que es requerido para la activación de otros genes simbióticos (Figura 1) (Smit, *et al*., 2005; Oldroyd & Downie, 2008; Hirsch, 2009). NSP1 contiene sitios de unión a DNA que no posee NSP2, por lo que se requiere de la interacción de ambos para asociarse a los promotores de los genes simbióticos (Hirsch, 2009). El complejo transcripcional NSP1/NSP2 promueve la expresión de *NIN* (Nodule Inception Protein), y *ERN* (Ets2 Repressor Factor), (Schauser *et al*, 1999; Borisov, *et al*., 2003; Andriankaja, *et al*., 2007; Middleton, *et al*., 2007; Hirsch, *et al*., 2009) los cuales codifican para factores de transcripción con funciones específicas para la nodulación (Stracke, *et al*., 2002; Marsh, *et al*., 2007; Middleton, *et al*., 2007; Cerri, *et al*., 2012). Por lo tanto la percepción de los FNs activa una red transcripcional, cuya acción coordinada es necesaria para activar cambios morfológicos en la leguminosa para que pueda llevar a cabo el proceso infección del rizobio, la formación del nódulo y subsecuente colonización por los rizobios.

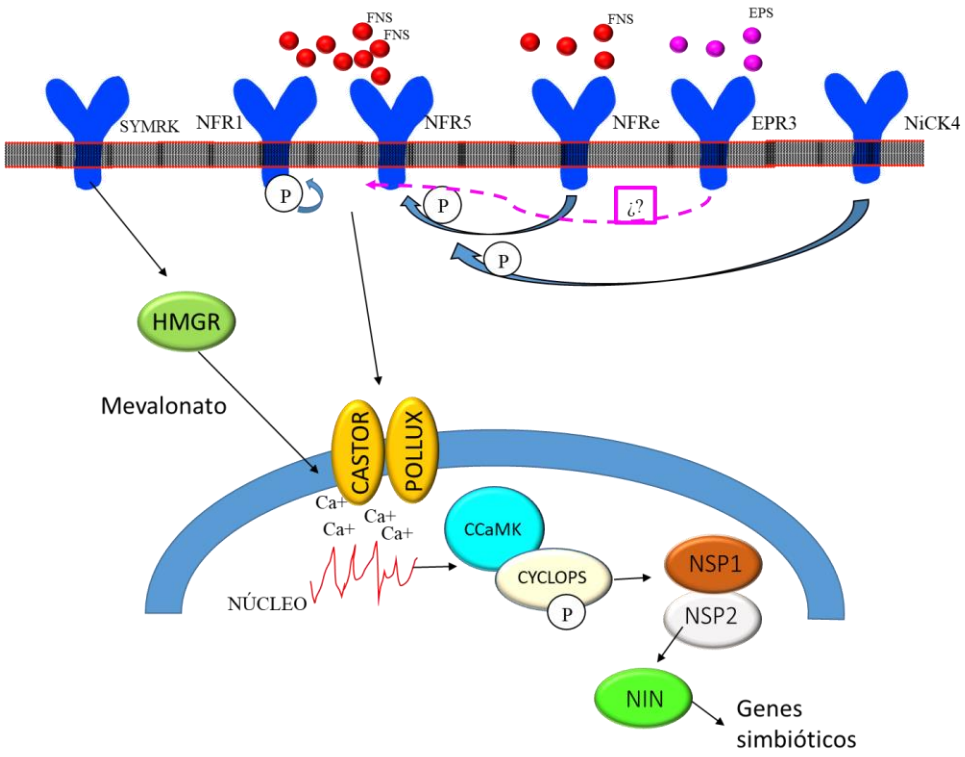

Figura 1. Esquema de la cascada de señalización que ocurre al percibirse los FNs. Al ser sensados los FNs por los receptores NFR1 y NFR5, adicionalmente de los otros receptores que interactúan con NFR5, se comienza a mandar la señal para que se activen los genes simbióticos que permitan la infección del rizobio y la organogénesis del nódulo.

1.2.4 Cascada de señalización: cambios morfológicos

Una vez que se han percibido los FNs y la subsecuente activación de los eventos moleculares descritos anteriormente, se inician una serie de cambios morfológicos en los pelos radicales (Cardenas, et a., 1998; de Ruijter, *et al*., 1998) y en las células del cortex de la raíz (Calvert, *et al*., 1984; Mathews, *et al*., 1989) que son necesarios para la formación del nódulo y albergar al rizobio compatible.

Uno de los primeros cambios morfológicos ocurre cuando el rizobio se aproxima a la punta del pelo radical en crecimiento y la decodificación de los FNs hace que el crecimiento polar del pelo radicular se re-direccione (Oldroyd, *et al*., 2011). El

crecimiento se interrumpe a lo largo del mismo y ocurre una hinchazón en la punta (Esseling, *et al*., 2003). Para que ocurran estas modificaciones en el crecimiento polar del pelo radicular, son necesarios rearreglos en su citoesqueleto. Se ha demostrado que las proteínas LIN (LUMPY INFECTIONS) y VAPYRIN (VPY) participan en este proceso (Kiss, *et al*., 2009; Murray, *et al*., 2011). Asimismo, se ha observado que estos rearreglos del citoesqueleto también son necesarios para reubicar al núcleo y se permita el paso del rizobio hacia el interior del pelo radicular (Liu, *et al*., 2019).

Posterior a los rearreglos del citoesqueleto y a la hinchazón del ápice del pelo radicular, su crecimiento se reanuda y ocasionando que el pelo se doble sobre si, provocando un encorvamiento (conocido como curling) que es crucial para atrapar al rizobio en la cámara de infección (Cardenas, *et al*., 1998; de Ruijter, *et al*., 1998; Esseling, *et al*., 2003; Fournier, *et al*., 2015). El rizobio forma una bolsa de infección (Gertus, 2005) donde continúa replicándose y produciendo FNs (Gage, 2004; Held, *et al*., 2010) cuya detección activa las cascadas de señalización necesarias para otro arreglo del citoesqueleto y la activación de enzimas que modifican la pared celular (Cai, *et al*., 2018; Malolepszy, *et al*., 2018). Estas respuestas moleculares son necesarias para formar una invaginación tubular de la membrana plasmática, denominada hilo de infección (HI), el cual se extenderá desde la cámara de infección hasta las células corticales de la raíz donde se desarrollará el nódulo (Gage, 2004; Oldroyd & Downie, 2004; Miwa, *et al*., 2006; Oldroyd, *et al*., 2011). Los receptores encargados de reconocer a los FNs (*SYMRK/DMI2*) siguen presentes a lo largo de la membrana que forma el hilo de infección (Limpens, *et al*., 2005), por lo que estos genes juegan un papel importante en la infección del rizobio (Ferguson, *et al*., 2010). A través de este HI la bacteria entra a la leguminosa (sin penetrar la membrana plasmática) por lo que el HI facilita la penetración del rizobio (Ferguson, *et al*., 2010). Sin embargo, es importante mencionar que esta no es la única forma de entrada de los rizobios, también se ha documentado que en algunas leguminosas (e.g., soya) el rizobio ingresa a la raíz mediante aperturas que se dan en las zonas de la epidermis donde emergen las raíces laterales y, por lo tanto, no se forma el HI (Gage, 2004).

Otro receptor que se encuentra alrededor de los hilos de infección es NFR1/LYK3 el cual una vez que se establece la infección, se asocia con proteínas que se asocian a balsas lipídicas denominadas Flotilinas2 y 4 (Flotilin-like, FLOT4) (Haney, *et al*., 2011). FLOT2 y FLOT4 participan en el rearreglo del citoesqueleto y permiten la formación del hilo de infección (Kioka, *et al*., 2002; Langhorst, *et al*., 2008). Las flotilinas también ayudan a reclutar proteínas involucradas en la señalización para la formación del nódulo. De tal manera que el HI se extiende hasta las células corticales del nódulo en desarrollo (Oldroyd, *et al*., 2011). El HI es la estructura mediante la cual el rizobio entra al citoplasma de las células de la planta y una vez liberado, se le rodea de una membrana para que se diferencie a bacteroide y formar el simbiosoma (Figura 2) (Udvardi & Day, 1997; Oldroyd & Downie, 2004).

En el nódulo, algunas células están invadidas por el bacteroide, las cuales se encargarán de fijar el N_2 a amoniaco y exportarlo por difusión hacia las células no infectadas del nódulo. A su vez, las células no infectadas brindan el aporte de nutriente necesario para el bacteroide (Newcomb, *et al*., 1979; Calvert, *et al*., 1984; Udvardi & Day, 1997). La asimilación de amoniaco se da por la via GS-GOGAT, donde el amoniaco pasa a glutamina y posteriormente a glutamato (Cullimore & Bennett, 1988; Atkins, 1991; Vance & Heichel, 1991). Las enzimas de esta via son muy activas en el nódulo, de tal manera que el amoniaco se mantiene a bajas concentraciones en el citoplasma de las células vegetales (Streeter, 1989). Por lo que el bacteroide está suplementando constantemente amoniaco. El nitrógeno asimilado se trasloca a otras partes de la planta y dependiendo del tipo de leguminosa, se exportan en amidas (clima templado) o ureidos (clima tropical) (Atkins, 1991; Schubert, 1986).

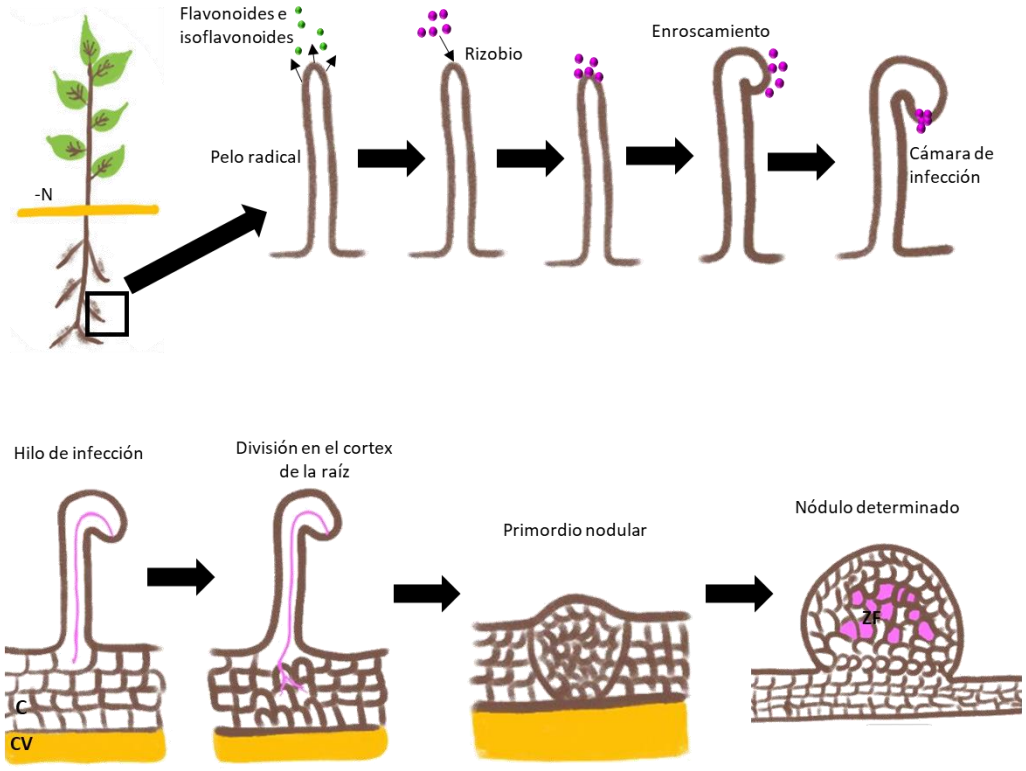

Figura 2. Simbios entre leguminosas y rizobios. La baja disponibilidad de nitrógeno en el suelo induce la excreción de flavonoides e isoflavonoides hacia la rizosfera. Los rizobios son atraídos a los pelos radicales y al ser reconocidos los FNs comienza el encorvamiento del pelo radical para formar la cámara de infección. Posteriormente se forma el hilo de infección y se elenga hasta las células del cortex. Las céluas del cortex se dividen y comienza la formación del primordio nodular hasta llegar a la formación del nódulo, el cual posee los bacteroides que se encargan de la fijación simbiótica de nitrógeno. C. Cortex de la raíz. CV: Cilindro vascular. ZF: Zona de fijación.

1.3 miRNAs en la simbiosis.

Además de la participación de diferentes factores de transcripción en la regulación de la simbiosis leguminosa-rizobio, también se ha demostrado que diversos RNAs pequeños no codificantes [e.g., microRNAs (miRNAs)] juegan un papel importante en el establecimiento de esta simbiosis (Bazin *et al*., 2012).

Los miRNAs en plantas son de una longitud promedio de 21 nucleótidos (Llave, *et al*., 2002; Palatnik, *et al*., 2003;) y actúan sobre secuencias de RNA mensajeros (mRNAs) (Mallory & Vaucheret, 2006), regulando su expresión mediante el

silenciamiento post-transcripcional (PTGS, post-transcriptional gene silencing) y silenciamiento transcripcional (TGS, transcriptional gene silencing) (Jones-Rhoades, *et al*., 2006).

Los miRNAs participan en practicamente cada una de las etapas de desarrollo y crecimiento de las plantas. Además, los miRNAs también regulan diversos procesos que les permiten a las plantas adaptarse al ambiente (Jones-Rhoades, *et al*., 2006; Mallory & Vaucheret, 2006). En el caso de la simbiosis leguminosa-rizobio se ha demostrado la participación de diferentes miRNAs, particularmente en el proceso del desarrollo y diferenciación del nódulo (Bazin, *et al*., 2012). Por ejemplo, se ha reportado que la acumulación del miRNA miR171 puede ser dependiente de la detección de los FNs (Branscheid, *et al*., 2011; De Luis Margarit, 2010). Además, se ha demostrado que miR171 se acumula tanto en raíces de diferentes leguminosas inoculadas con rizobio como en diferentes etapas de desarrollo del nódulo, y que tiene como blanco a *NSP2*, el cual codifica para parte del heterodimero NSP2/NPS1 necesario para activar la expresión de genes simbióticos involucrados en el proceso de infección del rizobio y formación del rizobio (De Luis Margarit, 2010; Branscheid, *et al*., 2011; Devers, *et al*., 2011).

Otros miRNA que también están involucrados en la formación y desarrollo del nódulo son miR164, miR169 y miR166. El primero también esta involucrado en la formación de raíces laterales y su blanco es el activador transcripcional NAC1, la sobreexpresión de este miR164 resulta en una reducción del número de nódulos (Subramanian, *et al*., 2008; D´haeseleer, *et al*., 2011). Con respecto a miR169, se ha observado que este miRNA regula al gen *MtHAP2-1*, el cual participa en la diferenciación de las células del nódulo y se expresa en la zona de infección en *M. truncatula* (Combier, *et al*., 2006; Boualem, *et al*., 2008). Por otro, lado se ha reportado que el miR166 presenta una expresión elevada en nódulos en desarrollo (nódulos de 3 días), la cual va disminuyendo conforme avanza la edad del nódulo (Boualem, *et al*., 2008). También, se ha observado que la sobreexpersión de miR166 repercute en el número de nódulos y de raíces laterales en *M. truncatula* (Boualem, *et al*., 2008). Por lo anterior, estos miRNAs controlan la nodulación

reprimiendo factores de transcripción claves para este proceso (Combier, *et al*., 2006; Boualem, *et al*., 2008).

Otros miRNAs reportados que incrementan su expresión después de que la leguminosa es inoculada con su simbionte son: miR393 (Navarro, *et al*., 2006; Subramanian, *et al*., 2008), miR482, miR1507 (Li, *et al*., 2010), miR168, miR159, miR171, miR482, miR398, miR172 y miRNA172c (Subramanian, *et al*., 2008; Formey, *et al*., 2016). En el caso de miRNA172c se ha reportado que regula la expresión del factor de expresión AP2-1 y que tiene un papel en la deformación del pelo radical en *Phaseolus vulgaris* (Nova-Franco, *et al*., 2015). Recientemente, se reportó que en *G. max* miR172c regula la expresión del gen *Nodule Number Control1*, el cual es un represor de la expresión de los genes *GmRIC1* y *GmRIC2*, los cuales participan en la vía de señalización Autoregulación de la Nodulación, la cual regula el número de nódulos en esta leguminosa (Wang *et al*., 2019).

Es evidente que los miRNAS tienen un papel en la regulación de la simbiosis leguminosa-rizobio (Bazin, *et al*., 2012). Sin embargo, para que los miRNAs puedan cumplir con sus funciones regulatorias es necesario que sean incorporados al complejo de silenciamiento (RISC) para que puedan reprimir a sus genes blanco (Jones-Rhoades, *et al*., 2006). El componente crítico del RISC son las proteínas Argonautas (AGOs), las cuales son cruciales para procesar postranscripcional o traduccionalmente los RNAm (Zhang, *et al*., 2015; Palatnik, *et al*., 2003; Llave, *et al*., 2002).

1.4 Proteínas Argonauta

Las proteínas AGO reciben su nombre de la planta mutante de *Arabidopisis thaliana* debido a que sus hojas adoptaban la forma del calamar *Argonauta* (Bohmert, *et al*., 1998). Estas proteínas están presentes en bacterias, arqueas y eucariotas, lo que indica un origen ancestral (Cerutti & Casas-Mollano, 2006). La familia de las proteínas AGO se subdivide en dos: AGO y PIWI, aunque este último no se encuentra en plantas (Carmell, *et al*., 2002; Cerutti & Casas-Mollano, 2006;

Hutvagner & Simard, 2008). Además de la existencia de una tercera familia que solo se ha encontrado en *Caenorhabditis elegans* (Yigit, *et al*., 2006). Las proteínas AGO pesan entre 90-100 kDa y consisten de un domino N-terminal variable (N), un dominio conservado PAZ (PIWI-ARGONAUTE-ZWILLE), un MID (middle), un dominio PIWI y un dominio conservado C-terminal (Tolia & Joshua-Tor, 2007; Hutvagner & Simard, 2008; Vaucheret, 2008) (Figura 3a). El dominio N es el menos estudiado (Fan & Qi, 2016), y se sugiere que ayuda a la unión entre el RNA pequeño (sRNA, small RNA) y el RNA blanco (Wang, *et al*., 2009). El dominio PAZ une oligonucleótido/oligosacárido (OB), lo que permite la unión a una hebra simple de ácidos nucleicos (Lingel, *et al*., 2003; Song, *et al*., 2003; Yan, *et al*., 2003) y reconocer el extremo 3' de los sRNAs (Tolia & Joshua-Tor, 2007; Hutvagner & Simard, 2008). El dominio MID posee un loop de especificidad para reconocer el nucleótido 5´del sRNA, lo que define la preferencia de unión de las AGO con los diferentes sRNAs acorde al nucleótido en esta posición (Tolia & Joshua-Tor, 2007; Mi, *et al*., 2008; Montgomery, *et al*., 2008; Takeda, *et al*., 2008; Hutvagner & Simard, 2008; Frank, *et al*., 2010; 2012). El dominio PIWI tiene una estructura similar a la de una RNAsa H y se le atribuye actividad endonucleasa, el motivo con actividad catalítica que corta el blanco de RNAm es de DDH/D (Asp-Asp-His/Asp) (Liu, *et al*., 2004; Song, *et al*., 2004; Rivas, *et al*., 2005). Sin embargo, no todas las AGO han sido reportadas con actividad endonucleasa (Nowotny, *et al*., 2005; Saito, *et al*., 2006; Vaucheret, 2008). La interface MID-PIWI contiene una hendidura que ayuda a que se ancle el fosfato 5´ del sRNAs (Parker, *et al*., 2005) (Figura 3b).

1.4.1 RISC

Las proteína AGO son componentes del complejo de silenciamiento inducido por RNA (RISC, por sus siglas en inglés) junto con diferentes RNAs pequeños no codificantes, incluídos los miRNAs (Song, *et al*., 2004). El complejo RISC se forma en dos pasos, primero se lleva a cabo la selección de la hebra doble de sRNAs entre las proteínas AGO y la selección de la hebra guía en cada AGO (Takeda, *et al*.,

2008). La hebra doble de sRNA que se asocia con la proteína AGO es conocida como la “hebra guía” y la otra es la “hebra pasajera”. La hebra guía se selecciona termodinámicamente (Khvorova, *et al*., 2003; Schwarz, *et al*., 2003; Jones-Rhoades, *et al*., 2006; Takeda, *et al*., 2008). El complejo de AGO con la hebra guía se le conoce como el RISC maduro y puede dirigirse a sus blancos (mRNA) en lo que se conoce como la secuencia semilla (Lewis, *et al*., 2003). Mientras que la hebra pasajera es desplazada y degradada (Fang & Qi, 2016) (Figura 3c)

Diversos estudios en plantas muestran que existe un alto grado de complementariedad entre los miRNAs y sus mRNAs blanco, lo que lleva al corte del mRNA (Llave, *et al*., 2002; Rhoades, *et al*., 2002; Tang, *et al*., 2003). Aunque también se ha llegado a ver en la capacidad de reprimir la traducción (Aukerman & Sakai, 2003; Chen, 2004; Gandikota, *et al*., 2007; Brodersen, *et al*., 2008; Li, *et al*., 2013).

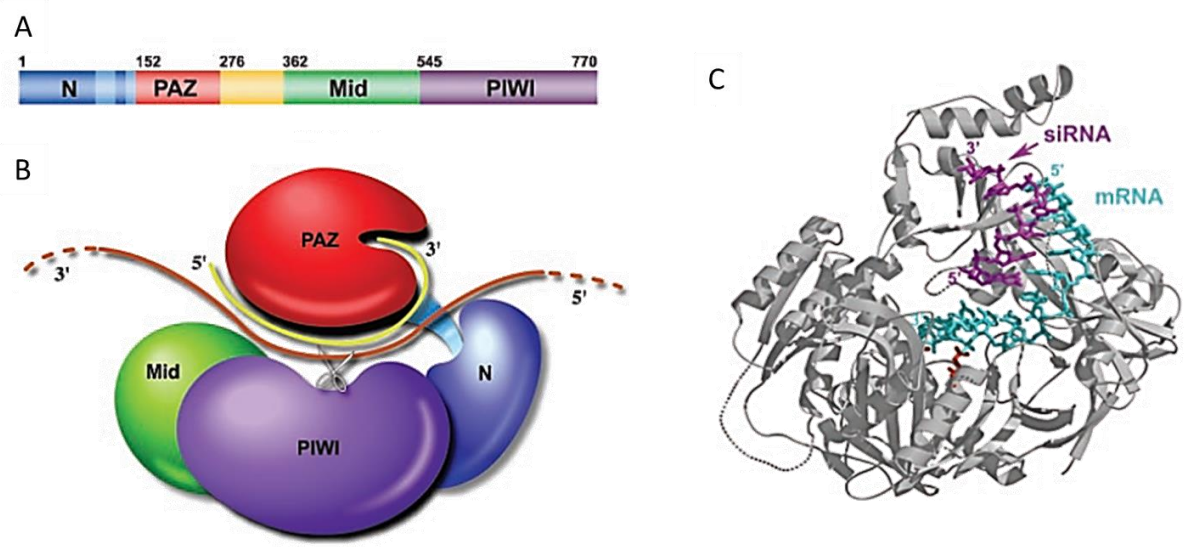

Figura 3. Proteína Argonauta. A) Diagrama esquemático de los dominios de la proteína AGO. Dominio N-terminal (azul), dominio PAZ (rojo), dominio MID (verde) y dominio PIWI (morado).

B) Modelo de corte del mRNA guiado por un sRNA. Los dominios siguen el patrón de color del panel A. El RNA guía (hebra amarilla) se une a la hendidura del dominio PAZ, el mRNA (hebra roja) complementario es cortado por el sitio activo del dominio PIWI (representado por tijeras) y se da el corte del mRNA. C) Modelo de interacción de la hebra guía (hebra morada) interaccionando con el

dominio PAZ, la hebra de mRNA (hebra azul) se extiende y centra a lo largo del surco hacia el sitio activo del dominio PIWI (zona roja). Modificado de Song, *et al*., 2004.

1.4.2 Funciones de las proteínas AGO.

Las AGO tienen diferentes funciones, por ejemplo, participan en la regulación de la expresión genética a nivel post-transcripcional (escisión del mRNA/inhibición de la traducción) y a nivel transcripcional en la metilación del DNA (Law & Jacobsen, 2010; Rogers & Chen, 2013). Se sabe que esta familia de proteínas se ha conservado durante la evolución y se ha diversificado sus funciones en las plantas (Fang & Qi, *et al*., 2016). Por lo que sus funciones bioquímicas junto con sus patrones de expresión espacio-temporales pudieron llevar a la especialización de AGO en sus funciones biológicas (Fang & Qi, 2016).

En *A. thaliana* existen 10 proteinas AGO (Carmell, *et al*., 2002; Morel, *et al*., 2002; Vaucheret, 2008). Sin embargo, el número de proteínas que componen esta familia varia de acuerdo al organismo (Zhang, *et al*., 2015). Por ejemplo, en *Populus thichocarpa* hay 15 proteínas AGO (Zhao, *et al*., 2015), en maíz 17 (Qian, *et al*., 2011; Zhai, *et al*., 2014), en arroz 19 (Kapoor, *et al*., 2008), y 21 en soya (Liu, *et al*., 2014). Basándose en las relaciones filogéneticas realizadas en *A. thaliana* se dividen en 3 clados: I) AGO1/5/10, II) AGO 2/3/7 y III) AGO 4/6/8/9 (Morel, *et al*., 2002; Vaucheret, 2008; Zhang, *et al*., 2015, Fang & Qi, 2016). Las proteínas AGO de los clados II y III han sido poco estudiadas; sin embargo, se sabe que las proteínas AGO7 y AGO2 que pertenecen al clado II participan en la generación de un tipo de RNAs pequeños, como los tasiRNAs (trans-acting small interference) los cuales son formados cuando AGO7 se une a miR390 (Montgomery, *et al*., 2008). Por su parte AGO2, participa en la defensa contra virus y se ha visto que tiende a incrementarse ante la presencia de bacterias patógenas (Zhang, *et al*., 2011; Fang & Qi, 2016).

En el clado III AGO 4 participa en el silenciamiento transcripcional a través de la metilación del DNA (Vaucheret, 2008; Fang & Qi, 2016) y se expresa de forma

ubicua en toda la planta (Zheng, *et al*., 2007; Havecker, *et al*., 2010; Eun, *et al*., 2011). AGO6 tiene actividad redundante con AGO4 (Zheng, *et al*., 2007) y se expresa tejidos donde hay células en división pero no maduras (Zheng, *et al*., 2007; Havecker, *et al*., 2010; Eun, *et al*., 2011). AGO9 participa en la formación del gameto femenino (Olmedo, *et al*., 2010).

En el clado I, se ha reportado que AGO10 se une a miRNAs específicos, como mir165/166 (Zhu, *et al*., 2011; Wu, *et al*., 2015) y se sabe que participa en la determinación del meristemo floral (Ji, *et al*., 2011). La proteína AGO1 es la mejor caracterizada de todos los clados. Se ha descrito que interviene en el silenciamiento mediado por RNA (Fagard, *et al*., 2000), asociándose preferentemente con sRNAs que poseen un 5´uridina (Montgomery, *et al*., 2008; Takeda, *et al*., 2008; Boutet, *et al*., 2003). Participa en la defensa contra virus (Vaucheret, 2008; Morel, *et al*., 2002; Zhang, *et al*., 2006; Qu, *et al*., 2008; Takeda, *et al*., 2008; Wang, *et al*., 2011) y recientemente se ha descrito su participación en la simbiosis regulando genes involucrados con la infección por rizobio y el desarrollo del nódulo (Ren, *et al.,* 2019).

El segundo parálogo mas cercano a AGO1 es AGO5 (Vaucheret, 2008). En *A. thaliana* se ha reportado que AGO5 se expresa en las células somáticas de la megaespora (Vacheret, 2008; Tucker, *et al*., 2012) y durante la esporogénesis en arroz (Nonomura, *et al*., 2007). En las leguminosas se sabe que participa en la pigmentación de las semillas de soya (Cho, *et al*., 2017). Al asociarse preferentemente con sRNAs que poseen un 5´C (Takeda, *et al*., 2008), se ha propuesto que puede interaccionar con miR169 en *M. truncatula* y participar en el desarrollo del nódulo (Combier, *et al*., 2006). Otra evidencia de su participación en la interacción con microrganismos es que, tanto en *A. thaliana* como en soya se acumula al interaccionar con virus (Brosseau & Moffett, 2015; Valdés-López, *et al*., 2011).

El interés que mostramos en AGO5 deriva primero de la evidencia del incremento de esta proteína ante la presencia de virus, los cual indica que AGO5 participa en la respuesta de la planta en las interacciones con patógenos. Además, al revisar en las bases de datos el patrón de expresión de *AGO5*, se puede apreciar un incremento en su expresión en las raíces y nódulos de las leguminosas, no

encontrando esta tendencia en *A. thaliana* donde su expresión solo se detecta en plantas en floración, debido a su expresión en la megaspora. Se ha visto que el tubo polínico y el hilo de infección tienen una regulación similar e incluso existen genes involucrados en el desarrollo del tubo polínico durante la floración, que fueron adaptados a los procesos simbióticos (Rodríguez-Llorente, *et al*., 2004).

La expansión de las AGO sugiere una diversificación funcional de las vías de regulación por sRNA (Zhang, *et al*., 2015; Fang & Qi, *et al*., 2016) y el estudio de los miRNAs involucrados en la regulación de la simbiosis ha sido el centro de varias investigaciones (Hoang, *et al*., 2020). Recientemente, en nuestro grupo de investigación se ha hipotetizado sobre la participación de las proteínas AGO en el proceso simbiótico y la importancia de considerarlas como nuevos reguladores de este proceso, puesto que a pesar del creciente número de miRNAs investigados en la simbiosis, poco énfasis se le ha dado a las proteínas AGO (Valdés-López *et al.,* 2019a, Anexo ll)

En este trabajo, mostramos evidencia experimental de que AGO5 es un componente de la regulación de la simbiosis entre leguminosas y rizobios, desde etapas tempranas de este proceso, hasta el desarrollo del órgano fijador de nitrógeno.

2. HIPOTESIS.

Las proteínas AGO y los microRNAs forman parte del complejo RISC. Las proteínas AGO pueden estar involucradas en la interacción simbiótica leguminosa-rizobio. En particular, AGO5 presenta un mayor nivel de expresión en las raíces y nódulos de las leguminosas. Por lo que, la proteína AGO5 participa en el control de las diferentes etapas moleculares que permiten el establecimiento de la simbiosis entre leguminosas y rizobios.

3. OBJETIVOS.

3.1 Objetivo General.

Determinar el papel de ARGONAUTA 5 en el establecimiento de la simbiosis entre leguminosas (*Glycine max* y *Phaselous* vulgaris) y rizobios.

3.2 Objetivos Particulares.

Determinar si AGO5 participa en el diálogo molecular entre leguminosas y rizobios.

Determinar si AGO5 participa en el desarrollo del nódulo.

Determinar si la expresión de *AGO5* se activa en células de la raíz involucradas en el diálogo molecular entre leguminosas y rizobios así como en la formación del nódulo.

4. MATERIALES Y MÉTODOS.

4.1 Material vegetal.

En este trabajo se utilizaron dos leguminosas: *P. vulgaris* (frijol) cultivar Negro Jamapa y *G. max* (soya) variedad Merril Williams 82.

Las semillas de frijol se desinfectaron sumergiéndolas en etanol al 70% durante 1 minuto. Inmediatamente, se sumergieron en cloro al 10% y se lavaron ocho veces con agua destilada estéril, teniendo una duración de 10 minutos con agitación por lavado. Las semillas desinfectadas se germinaron durante dos días a 25°C en cajas Petri con papel de germinación humedecido con agua destilada estéril.

Las semillas de soya se desinfectaron sumergiéndolas en etanol al 70% durante 1 minuto. Inmediatamente, se sumergieron en cloro al 10% y se lavaron seis veces con agua destilada estéril, teniendo una duración de un minuto por lavado con agitación. Las semillas desinfectadas se germinaron durante cinco días a 25°C y con un fotoperiodo de 16/8 horas en cajas Petri de 25cm x 25cm que contenían medio Fähraeus (1957) con bajo contenido de nitrógeno.

Después del tiempo establecido para la germinación, las semillas de frijol y soya se transfirieron a macetas con agrolita. Las plantas se mantuvieron en un fotoperiodo de 16/8 horas, en una temperatura de 25°C y regadas con agua/solución con bajo contenido de nitrógeno, para el desarrollo de las plantas y su uso en los diferentes experimentos.

4.2 Cultivo de rizobios.

Rhizobium tropici CIAT 899, simbionte de frijol, fue crecido durante dos días en medio sólido PY (5 gr/ peptona, 3 gr/L extracto de levadura, 18 gr/L agar bacteriológico) suplementado con 20 μg/ml de ácido nalidíxico.

Bradyrhizobium diazoefficiens USDA110, el simbionte de soya, fue crecido durante 4 días en medio YEM sólido (10 gr/L manitol, 0.2 gr/L $MgSO_4*7H_2O$, 0.1 gr/L NaCl, pH 7.0, 18 gr/L agar bacteriológico).

Los rizobios se mantuvieron a 30°C durante el tiempo establecido para su crecimiento. Para su uso como inoculo, el rizobio fue colectado de una caja fresca y se resuspendió en 50ml de agua destilada estéril (solución saturada).

4.3 Construcciones para la generación de raíces transgénicas:

4.3.1 Silenciamiento de *AGO5* mediante RNAi

Para generar la construcción de *AGO5*-RNAi se amplificó por PCR y con primers específicos una región de 150 pares de bases localizada en el 3´UTR de *AGO5* de frijol o soya (Cuadro 1). El fragmento amplificado se clono en el vector pENTR-D-TOPO (Thermo Fisher Scientific, Waltham, MA, USA) y se verificó que no existieran errores en su secuencia mediante secuenciación. El plásmido pENTR-*AGO5*-RNAi se recombinó en el vector binario pTDT-DC-RNAi que expresa el gen reportero *tdTomato* (Tandem Dimer Tomato, Valdés-López, *et al*., 2008). La orientación de la construcción de *AGO5*-RNAi se verifico mediante PCR (Polimerase Chain Reaction) utilizando los primers WRKY Intron-fwd y *AGO5*-rev de la leguminosa en cuestión (Cuadro 1).

Los plasmidos pTDT-DC-RNAi (empty vector), pTDT-PvAGO5RNAi, pTDT-GmAGO5RNAi se introdujeron mediante electroporación a *Agrobacterium rhizogenes* K599 para la generación de raíces transgénicas en frijol o soya.

Primer	Secuencia	Observaciones
Fw_PvAGO5RNAi	TCA GAA TCT GGT TCT GCA TCT GGA	RNAi contra PvAGO5
Rev_PvAGO5RNAi	CACC TTC AAT TTC AAC TGT AGG AAA	RNAi contra PvAGO5
Fw_GmAGO5RNAi	AAG TTC AGT TAT ATA CAA AGT TCA GTT	RNAi contra PvAGO5
Rev_GmAGO5RNAi	CACC CAA GCT GAC GGA ACA TTG CCT TTC	RNAi contra PvAGO5
WRKY Intron-fwd	CTT CTC CAA CCA CAG GAA TTC ATC	Revisar orientación de RNAi

Cuadro 1. Primers utilizados para la construcción del vector PTDT-*AGO5*RNAi en frijol y soya.

4.3.2 Fusion transcripcional del promotor de *AGO5* con el gen reportero *GUS*

Mediante PCR y usando los primers F_Pv Ago5 prom1, F_PvproAG5_1.8 y Rev_Pv Ago5 prom (Cuadro 2) se clonaron 1kb y 1.8 kb río arriba del codón de inicio de *AGO5* de frijol (Phvul.011G088200). Las regiones promotoras de 1kb y 1.8 kb se clonaron en el vector pENTR-D-TOPO (Thermo Fisher Scientific). Posteriormente los vectores de entrada conteniendo cada región promotora fueron recombinadas en el vector binario pKGWFS7 que contiene el marco de lectura abierta de los genes reporteros *GUS* y *eGFP*. Los plásmidos resultantes, promPv*AGO5*$_{1.8kb}$-*GUS* y promPv*AGO5*$_{1kb}$-*GUS*, se introdujeron por electroporación a *A. rhizogenes* K599 para la generación de raíces transgénicas.

Primer	Secuencia	Observaciones
F_Pv Ago5 prom1	CACC TTT TGA GAG ATT TTA ATT TAA GTT GG	Amplificación del promotor de *AGO5* de 1kb
F_PvproAG5_1.8	CACC TGG CTA TCG AAT GAA GTA TGG GA	Amplificación del promotor de *AGO5* de 1.8kb
Rev_Pv Ago5 prom	TCT GCG AGT GAG AGG TTT CAG AGA	Amplificación del promotor de *AGO5*

Cuadro 2. Primers utilizados para la clonación del promotor de *AGO5* en frijol.

4.4 Generación de raíces transgénicas.

Las diferentes construcciones en *A. rhizogenes* K599 se cultivaron en medio sólido LB (10g/L de peptona, 10g/L de NaCl, 5g/L de extracto de levadura) suplementado con 100ug/ml de espectinomicina. Las construcciones en *A. rhizogenes* se utilizaron para la generación de plantas compuestas, es decir, plantas en cuyo sistema radical se promoverá el desarrollo de raíces transgénicas.

Después de 3 días de que las plantas de soya y frijol, la infección con *A.rhizogenes* K599 se llevó a cabo diluyendo el contenido de una caja Petri, en 10ml de agua destilada estéril. Aproximadamente 0.5ml de la solución se inyectaron en el meristemo cotiledonar de las plantas y se les coloco una bolsa plástica para mantener la humedad. Después de 8 días de la infección se revisó en las plantas compuestas el desarrollo de las raíces transgénicas y se colocaron en los diferentes tratamientos.

Las plantas se mantuvieron a 25°C y con un fotoperiodo de 16/8 horas luz – oscuridad.

4.5. Relación simbiótica entre leguminosa-rizobio.

4.5.1. Expresión y acumulación de AGO5.

Plántulas de soya y frijol de dos días fueron colocadas en medio Fähraeus donde crecieron por otros dos días. Transcurrido ese tiempo se inoculó cada leguminosa con su simbionte (soya-*B. diazoefficiens*, frijol-*R. tropici*), manteniéndose en oscuridad y a temperatura ambiente. Las raíces se colectaron y congelaron en nitrógeno líquido a las 1, 3, 6, 8, 12, 24, y 48 horas post-inoculación (hpi). Adicionalmente, para evaluar los niveles de expresión en diferentes órganos y corroborar lo reportado en las bases de datos de Phytozome V.12.1 (https://phytozome.jgi.doe.gov/pz/portal.html), se colectaron hojas, raíces y nódulos de 25 días post-inoculación, se congelaron en nitrógeno líquido y almacenaron a -80°C.

La acumulación de la proteína AGO5 se realizó en plántulas de soya y frijol de 5 dias y crecidas en Medio Fähraeus siguiendo los mismos tiempos (hpi) anteriores. Se colectaron 300mg de raíces en los diferentes tiempos y se congelaron en nitrógeno líquido y se guardaron a -80°C.

Las muestras fueron maceradas en 500ul de buffer de extracción (50mM $Na_4P_2O_7$, 1mM Na_2MoO_4, 25mM NaCl, 10mM EDTA-Na, 0.5% PVP, 250nM sacarosa, 50mM HEPES, 5%glycerol, pH7.5) suplementado con inhibidor de proteasas (Sigma-Aldrich, St. Louis, MO, USA). La extracción se realizó en frío y el extracto se centrifugo a 13000 rpm durante 5 minutos a 4°C. Las proteínas fueron separadas por electroforesis en geles 10% SDS-PAGE, colocando 60 μgr de proteína por muestra y se transfirieron a una membrana de 0.2 μm nitrocelulosa (Amershan Protan, GE Healthcare Life Sciences, Pittsburgh, PA, USA) por electrotransferencia. La detección de la proteína se hizo con el anticuerpo anti-AGO5 (Agrisera, Vännäs, Swede; 1:1500), seguido de anticuerpo secundario de conejo policlonal conjugado con Pexosidasa (anti-IgG, Sigma-Aldrich, 1:5000).

4.5.2. Expresión de genes simbióticos.

El papel de AGO5 en las etapas tempranas de la simbiosis leguminosa-rizobio se determinó en las raíces transgénicas de soya y frijol, expresando la construcción del vector vacío (EV-RNAi) o la construcción de *AGO5*-RNAi. Las raíces transgénicas se inocularon por 1 hora con su simbionte (*Gm-Bj, Pv-R*t). Posteriormente las raíces transgénicas se colectaron en nitrógeno líquido y se almacenaron a -80°C.

El RNA total se extrajo mediante el kit ZR Plant MiniPrep (Zymo Research, Irvine, CA. USA) siguiendo las instrucciones del fabricante. Posteriormente, se removió el DNA genómico del RNA purificado utilizando la enzima DNaseI RNase-free (Thermo Fisher Scientific) acorde a las instrucciones del fabricante. Se usó de 1μg de RNA libré de DNA genómico para la síntesis de cDNA mediante la Thermo Scientific RevertAid Reverse Transcriptase (Thermo Fisher Scientific) siguiendo las instrucciones del fabricante. El cDNA obtenido se utilizó para la medición de la expresión de genes simbióticos mediante PCR en tiempo real (qRT-PCR) en el equipo Step-One qPCR thermocycler (Applied Biosystems, Foster, CA, USA). Los genes para normalizar los niveles de expresión (housekeeping) utilizados para soya fueron soya *Cons6* y *Cons16* y para frijol *PvActina*. Los genes simbióticos analizados fueron: soya, *GmAGO5, GmMATE, GmFbox, GmNYB, GmNIN;* y para frijol: *PvAGO5, PvCYCLOPS, PvNSP2, PvNIN, PvFLOTILLIN2* y *PvENOD40.* Las secuencias de los primers usados para este análisis transcripcional se muestran en el cuadro 3.

Primer	Secuencia	Observaciones
F_Gmcons6 R_Gmcons6	AGA TAG GGA AAT GGT GCA GGT CTA ATG GCA ATT GCA GCT CTC	Normalización de datos de soya por qRT-PCR
F_Gmcons16 R_Gmcons16	TTC TGG AGT TGG AGG ACA CTG GGC ATC TTA ACA GCA GAA GCA	Normalización de datos de soya por qRT-PCR.
F_GmAGO5 R_GmAGO5	AGG CTG TGG TAT GCT TTT GTG CTC ATA TCA ATA CCC CCA CCA	Revisar expresión de *AGO5* por qRT-PCR en soya
F_PvActina R_PvActina	TGC AGA AGG TGA GGA GAG TTG GGC AGA ATG AAC CAG TCA AAA	Normalización de datos de frijol por qRT-PCR
F_PvAgo5 R_PvAgo5	GAG CTC GTG ATA GTA CAG ATA GAA TAG CAC AAA TTA TTG GTT AAA ATC	Revisar expresión de *AGO5* por qRT-PCR en frijol
F_PvCYCLOPS R_PvCYCLOPS	TCC TTA CCA CAT TCT GCT GAG A CCA AGA GAT TCC AGA GGT TCA	Revisar expresión de *CYCLOPS* por qRT-PCR en frijol
F_PvNSP2 R_PvNSP2	GAC GGT TAT CGG GTA GAG GAG CGG AGG AAG AAG AAG TCC AAA	Revisar expresión de *NSP2* por qRT-PCR en frijol
F_PvNIN R_PvNIN	GGG AGA AGA GGC GTA CGA AG GTT GTG GGA CAC ACT CCG AT	Revisar expresión de *NIN* por qRT-PCR en frijol
F_PvFLOTILLIN2 R_PvFLOTILLIN2	GGA ACC CTG TCA GAC AAA ACA TTC ACG AAT CCA AAA CAA CC	Revisar expresión de *FLOT2* por qRT-PCR en frijol
F_PvENOD40 R_PvENOD40	GGG TCC TTA CCC CTC ACA CT TGT AGC CAA AGC CTC TCA TCC	Revisar expresión de *ENOD40* por qRT-PCR en frijol

Cuadro 3. Primers utilizados para el análisis de la expresión de genes simbióticos en raíces transgenicas RNAi de soya y frijol.

4.5.3 Deformación de pelos radicales.

Plantas compuestas de soya y frijol se colocaron en medio Fähraeus y se inocularon con su simbionte (*Glycine max-Bradyrhizobium diazoefficiensj, P.vulgaris-Rhizobium tropici*) por 48 horas. Después se seleccionaron las raíces transgénicas (EV-RNAi y *AGO5*-RNAi) y se tiñeron con azul de metileno.
Las raíces se observaron en un microscopio óptico de luz visible y se cuantificaron los pelos radicales. Las deformaciones efectivas y no efectivas fueron contabilizadas así como la presencia de hilos de infección.

4.5.4. Nódulos con baja expresión de AGO5.

Plantas compuestas de soya y frijol expresando el vector vacío (EV) o la construcción de *AGO5*-RNAi fueron inoculadas con su respectivo simbionte (*Gm-Bj, Pv-Rt*). Después de inoculadas se cortó la raíz principal y se colocaron en macetas donde se regaron con solución nutritiva –N (bajo aporte de nitrógeno) (Summerfield, *et al.,* 1997). Después de 45 días (soya) y 30 días (frijol) se cuantifico la cantidad de nódulos transgénicos.
Los nódulos de soya o frijol fueron colectados e inmediatamente fijados en solución fijadora FAA (formol-acético-alcohol) y colocados en un tren de deshidratación (etanol 30%, 50%, 70%, 90%, 96%, 100%, 100% y 100%, una hora en cada uno). Posteriormente se utilizó alcohol-xilol (75-25%, 50%-50%, 25%-50%, dos horas cada uno) hasta llegar a xilol. Después se mezcló xilol:paraplast 1:1 y se metió a la estufa a 56°C durante 3 días, adicionando paraplast cada día para no perder el volumen. Una vez hecha la infiltración, se procedió al corte de los tejidos con un micrótomo de mano y se obtuvieron cortes de 25μm que fueron teñidos con safranina alcohólica al 80%. Las preparaciones se observaron en el microscopio NIKON ECLIPSE E200 de campo claro y las fotografías se obtuvieron con el software NIS ELEMENTES BR 3.2.

4.6 Tinción de GUS en raíces para el análisis del promotor de *AGO5*.

EL análisis *in silico* del promotor de *AGO5* se realizó en frijol (*PvAGO5*) y en sus ortólogos en soya (*GmAGO5*) y *Medicago truncatula* (*MtAGO5*) para identificar los elementos regulatorios tipo *cis* relacionados con la simbiosis. Para este fin, se extrajeron las secuencias de DNA reportadas en Phytozome, que corresponden a 5kb río arriba del condon de inicio de la transcripción de *AGO5* de las tres leguminosas.

El análisis de los elementos *cis* se realizo en base a dos diferentes tamaños de secuencia (1kb y 1.8kb) de la región promotora, debido a que estas fueron las que se clonaron en frijol para su posterior expresión espacio-temporal. Los dos tamaños de secuencia (1kb y 1.8kb) de las tres leguminosas, fueron introducidos en la base de datos NEW PLACE (Higo, *et al.,* 1999) y de los elementos *cis* obtenidos se reportaron aquellos relacionados con algún evento simbiótico.

La expresión espacio-temporal de AGO5 se evaluó en plantas compuestas de frijol expresando el vector vació (EV-GUS) y las construcciones prom*PvAGO5*$_{1kb}$-*GUS,* prom*PvAGO5*$_{1.8kb}$-*GUS.*

Las plantas compuestas con raíces transgénicas fueron colocadas en medio Fähraeus durante dos días. Posteriormente se inocularon con agua (mock) o *R. tropici* (rizobio) y se obtuvo la cinética a la 1, 3, 6, 12, 24, y 48 hpi. Adicionalmente, otras plantas compuestas se les cortó la raíz principal y se inocularon con el rizobio. Estas raíces fueron colectadas a los 20 y 25 días.

Las raíces se infiltraron con solución de *GUS* e infiltradas al vació por 10 minutos. Las raíces se protegieron de la luz e incubaron a 36°C durante 2 horas. La actividad de GUS se observó bajo microscopio esteroscopico y óptico.

4.7 Bases de datos: expresión de AGO5 y análisis filogenético.

La comparación de la expresión de *AGO5* entre *Arabidopsis thaliana, Medicago truncatula, Glycine max* y *Phaseolus vulgaris* se hizo en base a la base de datos PHYTOZOME (https://phytozome.jgi.doe.gov/pz/portal.html).
La identificación de los miembros de la familia AGO se realizó mediante un BLAST utilizando las anotaciones del gen disponibles (Wm82.a2.v1, P. vulgaris v2.1 y Mt4.0v2). El BLAST se realizó en base a AGO5 de soya (Gm.11G190900). Los miembros de la familia se validaron utilizando Phytozome y HMMER (e-value $<e^{-100}$). En *Arabidopsis* se identificaron 10 miembros de la familia AGO, mientras que en soya, *Medicago* y frijol se identificaron 23, 20 y 14, respectivamente. La validación de las proteínas AGO se basó en la presencia de los dominios conservados (PIWI y PAZ).

5. RESULTADOS.

La mayoría de los resultados que se presentan en este trabajo se reportaron en Reyero-Saavedra, *et al.* (2017, Anexo l). Adicionalmente, la información y conocimiento generado en el desarrollo de este proyecto contribuyó a la publicación de una segunda publicación en la que se discute la importancia de las proteínas AGO en el proceso simbiótico (Valdés-López, *et al.,* 2019a, Anexo ll)

5.1 AGO5 se expresa preferentemente en raíces y nódulos de las leguminosas.

Analizando las bases de datos de expresión disponibles en Phytozome V.12.1, se observó que la expresión de *AGO5* incrementa en las raíces de leguminosas en comparación con la baja expresión (0.34) en *A. thaliana*. *AGO5* se expresa preferentemente en la raíz y el nódulo de las plantas de soya, frijol y *M. truncatula* (Figura 4). Mientras que en en *A. thaliana* su expresión solamente es detectada en la megaspora.

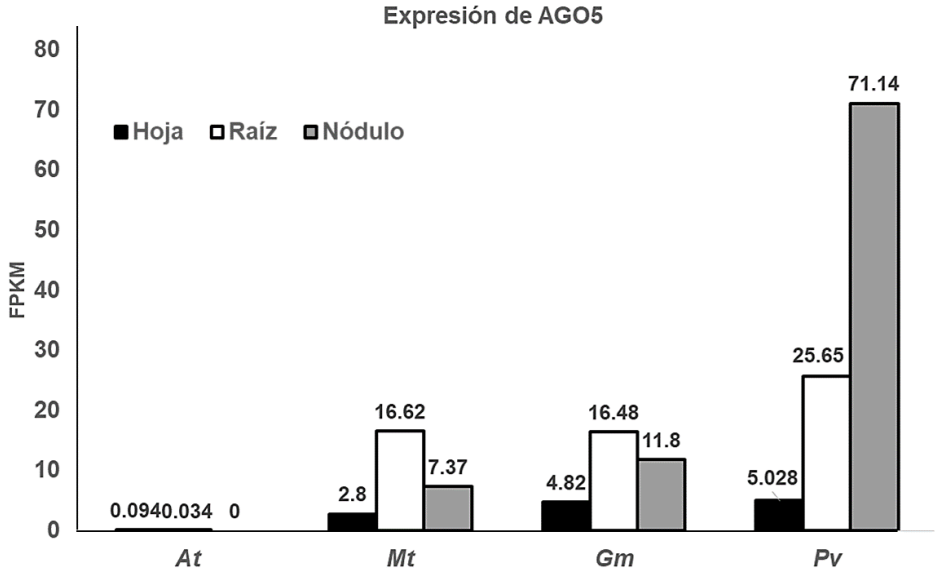

Figura 4. Expresión de *AGO5* en la base de datos Phytozome V.12.1 en *A. thaliana (AT2G27880.1*), *M. truncatula* (*Medtr4g056430.1*), *Glycine max* (*Glyma.11G190900.1)* y *Phaseolus vulgaris* (*Phvul.011G088200.1*). FPKM: Fragmentos de transcrito por Millón de secuencias mapeadas

El perfil de expresión de *AGO5* reportado en las bases de datos, permite formular la hipótesis de que *AGO5* podría jugar un papel en el establecimiento de la simbiosis entre leguminosas y rizobios. Previo a confirmar esta hipótesis de forma experimental, se confirmó que los genes *AGO5* de cada leguminosa, reportados en Phytozome, son los ortólogos de *AtAGO5*. Para ello se realizó un análisis filogenético con secuencias de proteínas de posibles ortólogos de AGO5 en diferentes especies de leguminosas, tales como *M. truncatula, G. max, P. vulgaris* y la planta modelo *A. thaliana*. Este análisis filogenético reveló que AGO5 de *M. truncatula*, soya y frijol presentan un 60% de identidad con respecto a AGO5 de *A. thaliana*. Confirmando además que las proteínas AGO5 de leguminosas pueden ser agrupadas en el Clado I de la familia AGO tal y como ha sido reportado para AtAGO5 (Figura 5a).

Para confirmar los resultados del análisis filogenético que indican que Phvul.011G088200.1 y Glyma.11G190900.1 son ortólogos de *AtAGO5*, se realizó un análisis de dominos de estas dos proetínas. Este análisis reveló que las proteínas AGO5 de soya y frijol contienen los dominios característicos de AGO5 (PAZ, MID, PIWI) excepto el dominio MID, el cuál no esta presente en frijol (Figura 5b). En conjunto, estos análisis bioinformáticos informan que Phvul.011G088200.1 y Glyma.11G190900.1 son los ortólogos de AtAGO5, sugiriendo que esta proteína AGO pudo haber sido neofuncionalizada durante el proceso de evolución de las leguminosas, jugando un papel determinante en el establecimiento de la simbiosis entre leguminosas y rizobios.

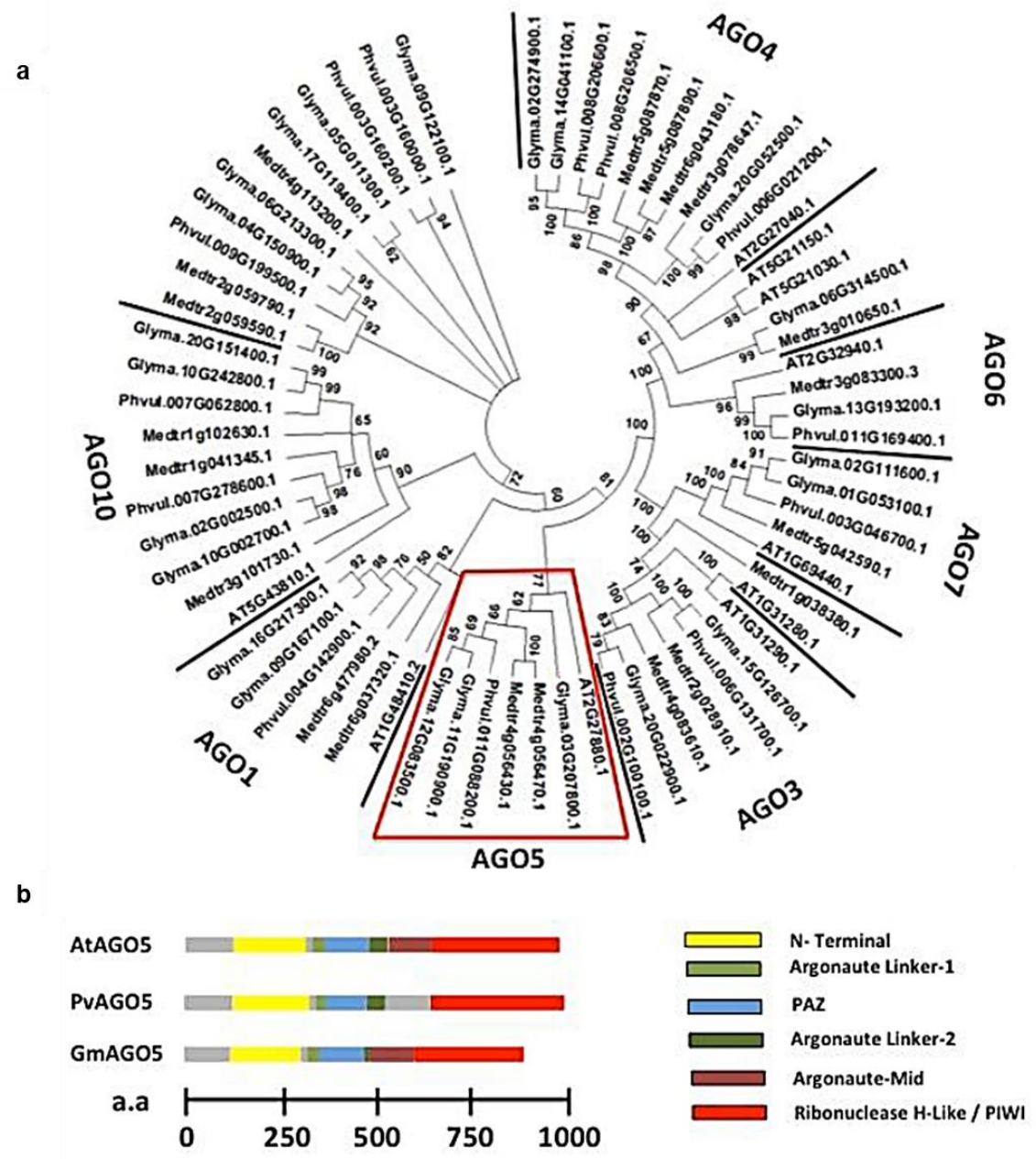

Figura 5. Análisis de AGO5 entre diferentes especies. A) Análisis filogenético de AGO5 entre *A. thaliana, M. truncatula, G. max* y *P vulgaris*. B) Análisis de dominios de las proteínas AGO5 de *Arabidopsis* (AtAGO5), soya (GmAGO5) y frijol (PvAGO5).

Una vez confirmado que los genes Phvul.011G088200.1 y Glyma.11G190900.1 codifican para un ortólogo de AtAGO5, se comprobaron los perfiles de expresión reportados en Phytozome mediante análisis transcripcionales por qRT-PCR. Como se observa en la Figura 6, la expresión de *AGO5* en hojas de soya y frijol es baja y se incrementa en las raíces y es mucho mayor en el nódulo. Este análisis además de confirmar lo reportado en Phytozome, refuerza la hipótesis de que AGO5 juega un papel en el establecimiento de la simbiosis leguminosa-rizobio.

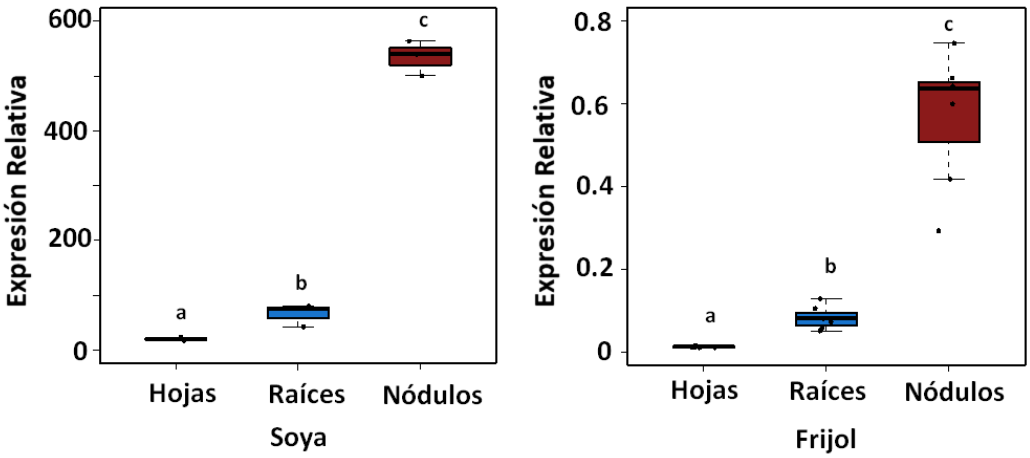

Figura 6. *AGO5* se expresa preferentemente en nódulos de soya y frijol. Los datos fueron analizados mediante un análisis de varianza (ANOVA), seguido de una prueba de Tukey. Las categorías estadísticas compartiendo la misma letra indican que no hubo diferencia significativa ($P<0.01$, $n=4$).

5.2 La expresión y acumulación de AGO5 responden a la presencia de rizobio.

La alta expresión de *AGO5* en raíz y nódulo sugiere que esta proteína AGO participa en el establecimiento de la simbiosis leguminosa-rizobio. Por lo anterior, se analizó la expresión de *AGO5* en las etapas tempranas del establecimiento de la simbiosis. El transcrito de *AGO5* fue cuantificado a diferentes tiempos en plantas inoculadas (1, 3, 6, 12, 24 y 48 horas después de la inoculación, hpi). En el caso de las plantas de soya, se observó un incremento del doble en la expresión del transcrito de *AGO5* en la primera hora del contacto con el simbionte (Figura 7a). Mientras que, en frijol se pudo observar la misma tendencia en las tres primeras horas de la interacción con rizobio (Figura 7b). Estos resultados indican que la expresión de *AGO5* se induce en las primeras horas de interacción con rizobios.

Para tener más evidencia sobre la respuesta de AGO5 a la presencia de rizobios, se analizó su acumulación en respuesta a *Rhizobium* mediante Western-Blot. En ambas leguminosas, se observó una acumulación de la proteína AGO5 durante la primera hora de la interacción con rizobios, lo cual es consistente con los resultados de expresión (Figura 7c, d). Sin embargo, en frijol hubo un segundo incremento en la acumulación de la proteína, el cual se mantuvo hasta las 24 horas (Figura 7 d). La diferencia en el patrón de incremento de la proteína entre las dos leguminosas y su respectivo simbionte, puede deberse a la variabilidad entre cada una de las simbiosis. Sin embargo, en ambas leguminosas la presencia del rizobio induce la expresión de *AGO5* y la acumulación de la proteína (traducción). En conjunto, estos datos sugieren que AGO5 participa en las etapas muy tempranas en la interacción simbiótica entre leguminosa y *Rhizobium.*

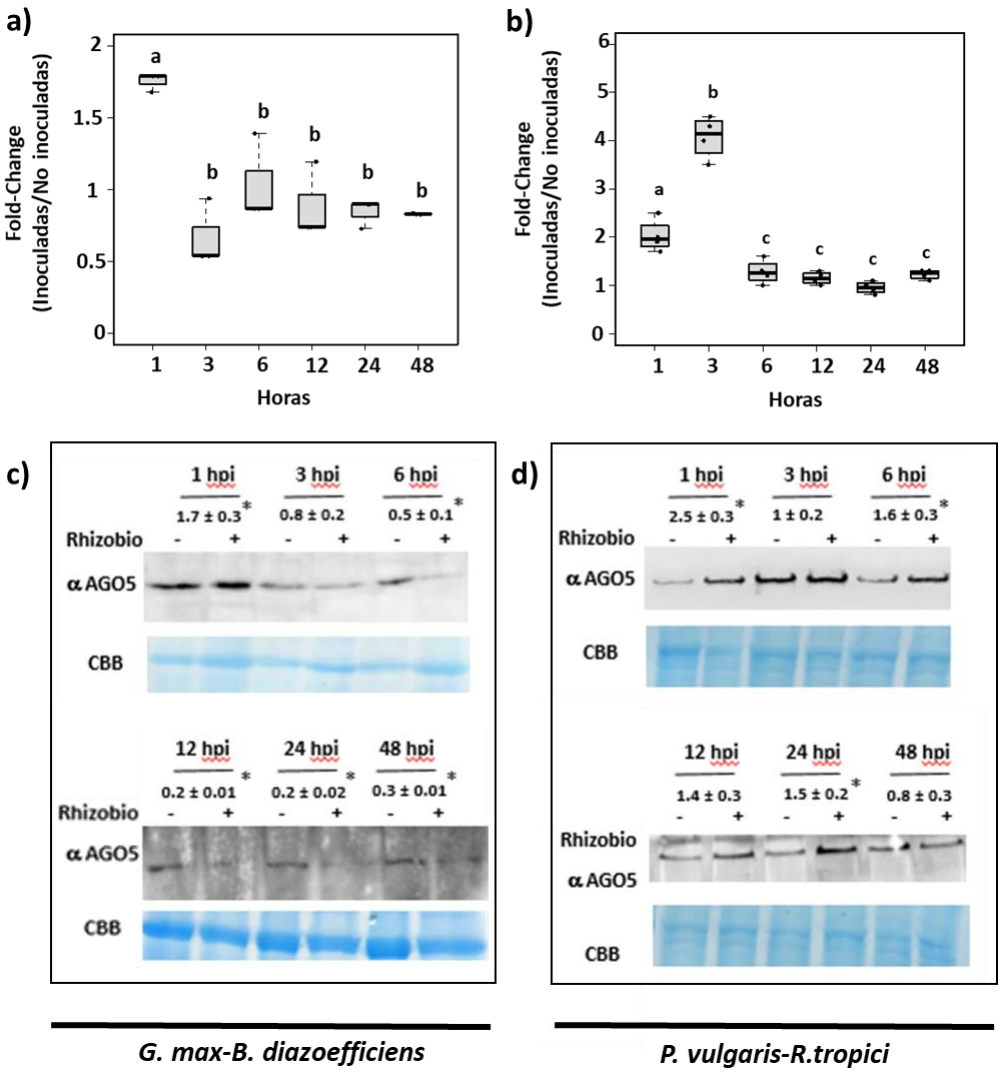

Figura 7. AGO 5 se induce en respuesta a rizobio. Expresión de *AGO5* durante las primeras horas de interacción con rizobio (horas post-inoculacion, hpi) en plantas de soya (a) y frijol (b). Los datos se analizaron mediante un análisis de varianza (ANOVA) seguida de una prueba de Tukey. ($P<0.01$, $n=4$). Acumulación de AGO5 en las primeras horas en respuesta a rizobio en soya (c) y frijol (d). La acumulación de AGO5 fue cuantificada por densitometría. Los asteriscos indican diferencia significativa acorde a un análisis de t-student ($p<0.01$, $n=3$).

5.3 El promotor de *AGO5* contiene elementos *cis* involucrados en etapas tempranas y en el desarrollo del nódulo.

El análisis *in silico* de los dos tamaños de secuencia (1kb y 1.8kb) de los elementos *cis* encontrados en el promotor *AGO5* se agruparon en cuatro categorías: l) En respuesta a Ca++/Calmodulina; ll) Relacionados a interacciones planta-patógeno; lll) Relacionados a la simbiosis; lV) Específico de la distribución del pelo radical (Figura 8a). El análisis mostró que en las leguminosas *M. truncatula* y *G. max* el número de elementos *cis* encontrados fue diferente entre los dos tamaños de secuencia analizados (1kb y 1.8kb). Mientras que en *P. vulgaris,* hubo siete elementos *cis* distribuidos en las categorías II, III y IV, los cuales se encontraron en las primeras 600 pares de bases rió arriba del codón de inicio, por lo que el número de elementos *cis* fue igual entre ambos tamaños de secuencias.

Los primeros dos elementos *cis* encontrados en frijol, se encuentra dentro de la categoría IV, en la posición -46pb y -104pb (Figura 8b). Posteriormente, en la posición -322pb existe un elemento (GT1-GMSCAM4) el cual responde a la interacción planta-patógeno (Campos-Soriano, *et al*., 2011). Finalmente, en la posición -579pb existen cuatro elementos *cis* cuya secuencia responde a nodulinas (NODCON1GM, NODCON2GM) y a los promotores que son activados en células infectadas de nódulos (OSE1ROOTNODULE, OSE2ROOTNODULE). Los resultados obtenidos del análisis de elementos *cis* indican que las regiones promotoras de *AGO5* de soya y frijol tienen elementos regulatorios que facilitan su expresión en respuesta a rizobios.

		1kb			1.8kb		
		Mt	Gm	Pv	Mt	Gm	Pv
I	CGCG-BOX	2			2		
II	GT1-GMSCAM4		2	1	2	3	1
	GCC-CORE						
	HSRENTHSR203J						
III	NODCON1GM	2	2		4	4	
	NODCON2GM	4	1	2	4	2	2
	OSE1ROOTNODULE	2	2		4	4	
	OSE2ROOTNODULE	4	1	2	4	2	2
IV	RHERPATEXPA7			2	1		2
	TOTAL ELEMENTOS	14	8	7	21	15	7

b)

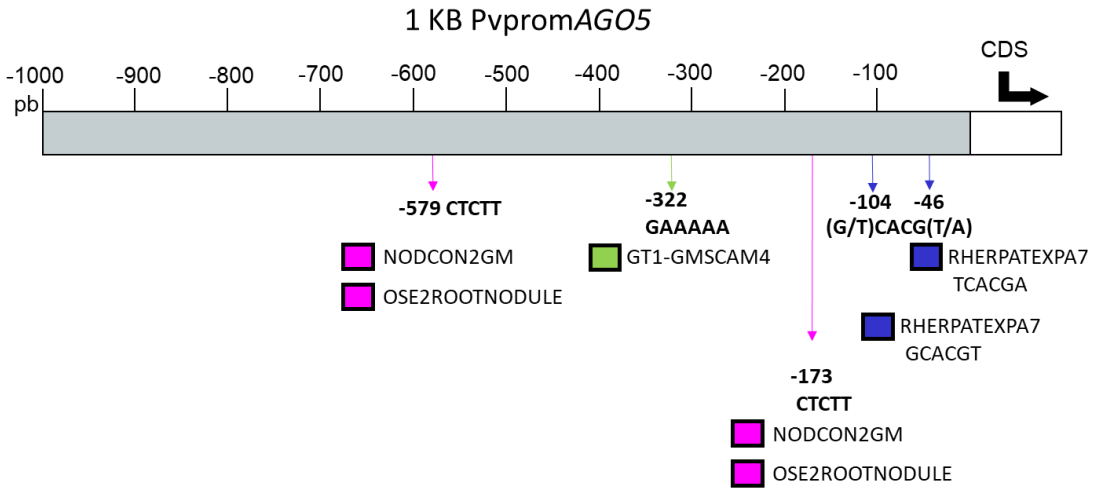

Figura 8. Elementos *cis* localizados en el promotor de *AGO5* en *M. truncatula* (Mt), *G. max* (Gm) y *P. vulgaris* (Pv). A) Elementos divididos en las categorías: I Relacionados con la respuesta a Ca++/Calmodulina (sin color); II Interacciones planta-patógeno (verde); III Relacionados con la simbiosis (rosa); IV Específico para la distribución del pelo radical (azul). Se indica el número de elementos *cis* por categoría por leguminosa. B) Posición y secuencia de los elementos *cis* en el promotor de 1 kb de AGO5 en frijol. Los colores corresponden a las categorías del panel anterior.

Para analizar la expresión espacio-temporal de *AGO5* mediante la actividad de GUS se utilizaron plantas compuestas de frijol con raíces transgénicas expresando la construcción prom*PvAGO5*$_{1kb}$-*GUS, Pv* prom*PvAGO5*$_{1.8kb}$-*GUS* o el control (EV-*GUS*). Los tiempos establecidos para el análisis fueron 1, 3, 5, 12, 24, y 48 horas

postinoculación (hpi), tanto en las raíces inoculadas con *Rhizobium tropici* como en las no inoculadas (agua).

Los resultados muestran que en las raíces control (EV-*GUS*) tratadas con agua o rizobio no hubo expresión de *GUS* (Figura 9a, b). Mientras que en las raíces transgénicas de frijol prom*PvAGO5*$_{1kb}$-*GUS* y prom*PvAGO5*$_{1.8kb}$-*GUS* hubo actividad de GUS en el cilindro vascular de la raíz durante la primer hora de tratamiento de las raíces inoculadas con agua o rizobio (Figura 9 c, d), esta expresión no se relaciono con la presencia de rizobio. La falta de expresión de GUS en el control indicó que la expresión de este, esta siendo regulada por el promotor de AGO5.

Los resultados obtenidos en la expresión espacio-temporal de las raíces transgénicas de frijol prom*PvAGO5*$_{1kb}$-*GUS* y prom*PvAGO5*$_{1.8kb}$-*GUS* son similares por lo que en los siguientes resultados de la cinéticas solo se hace referencia a prom*PvAGO5*$_{1kb}$-*GUS.*

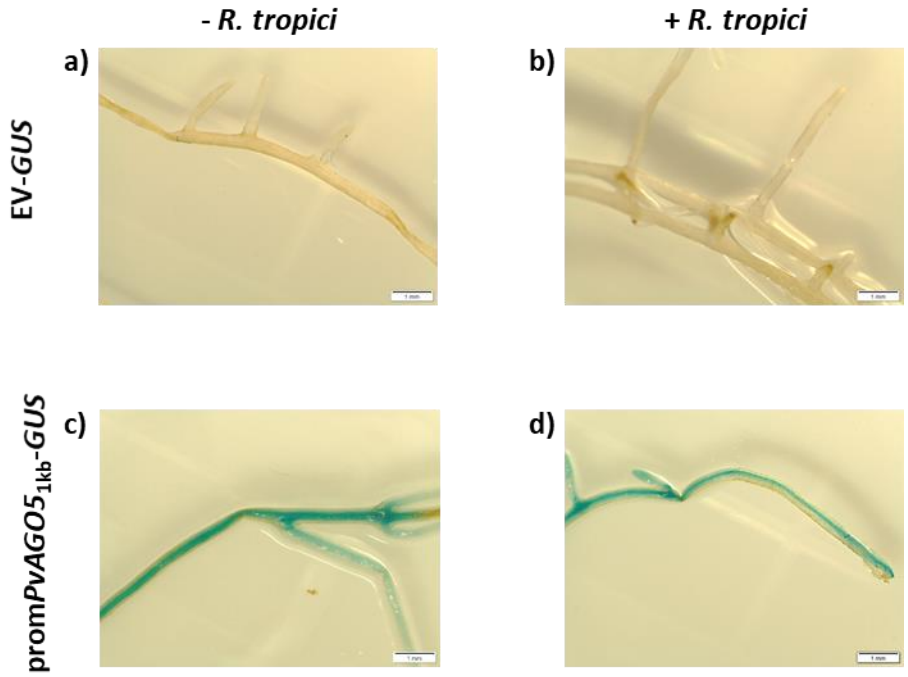

Figura 9. Raíces transgénicas de frijol sin inocular (a, c) e inoculadas con rizobio (b, d). Raíces control (EV-*GUS*) (a yb) y raíces prom*PvAGO5*$_{1kb}$-*GUS* (c y d).

En las 1ª y 3ª horas post inoculación (o hpi) de las raíces transgénicas de frijol que llevan la construcción prom*PvAGO5*$_{1kb}$-*GUS* no se observó actividad de GUS en los pelos radicales, ni su deformación (Figura 10 a, b). La expresión tisular de *AGO5* fue detectada a partir de 6hpi. Tiempo en el que se empezó a observar actividad de GUS en la punta de algunos pelos radicales. También se observó la tendencia a acumularse hacia la punta del pelo radical sin que exista aún deformación del pelo radical (Figura 10 c). La figura 10 muestra la cinetica de 1, 3 y 6hpi de las raíces que llevan la construcción prom*PvAGO5*$_{1kb}$-*GUS* inoculadas con rizobio. Las raíces inoculadas con agua mantienen la expresión en el cilindro vascular (Figura 9). Las raíces transgénicas con la construcción EV-*GUS* (control) inoculadas con agua y rizobio no se presentó actividad de GUS o deformación de pelos.

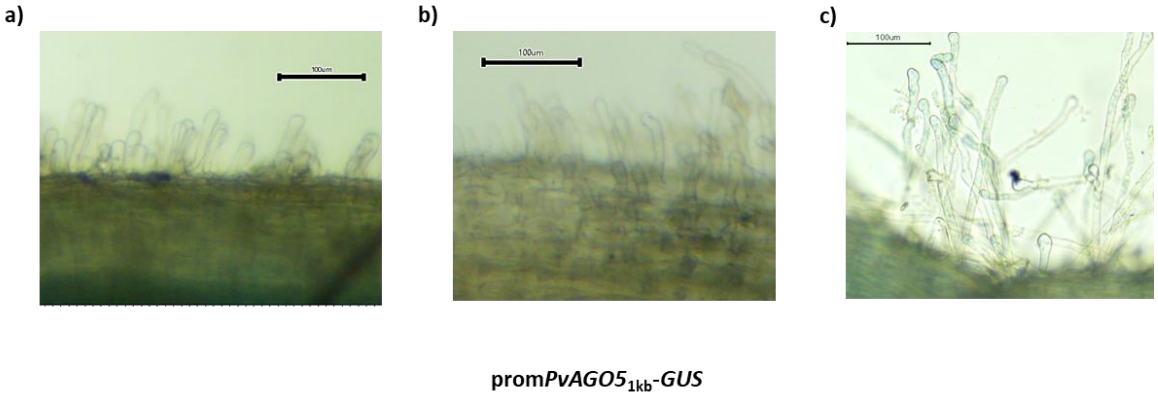

Figura 10. Raíces transgénicas de frijol que llevan la construcción prom*PvAGO5*$_{1kb}$-*GUS* inoculadas con *R. tropici*. a) 1hpi. b) 3hpi. c) 6 hpi.

A partir de las 12 hpi, se observó un incremento en la actividad de GUS en las raíces transgénicas de frijol prom*PvAGO5*$_{1kb}$-*GUS* y prom*PvAGO5*$_{1.8kb}$-*GUS.* Esta se localizó principalmente en la punta del pelo radical y acompaña a la deformación del mismo (Figura 11 a). Dicha acumulación se mantiene hasta las 48 horas en las raíces inoculadas con rizobio. Las raíces que no fueron inoculadas solo presentan actividad basal en el cilindro vascular, sin que exista actividad en la punta del pelo radical. Por lo tanto *AGO5* se expresa en los pelos radicales en respuesta a la presencia de rizobios. En las raíces control (EV*GUS*) inoculadas comienzan a presentarse deformaciones en respuesta a rizobio, sin que exista actividad de GUS (Figura 11 b).

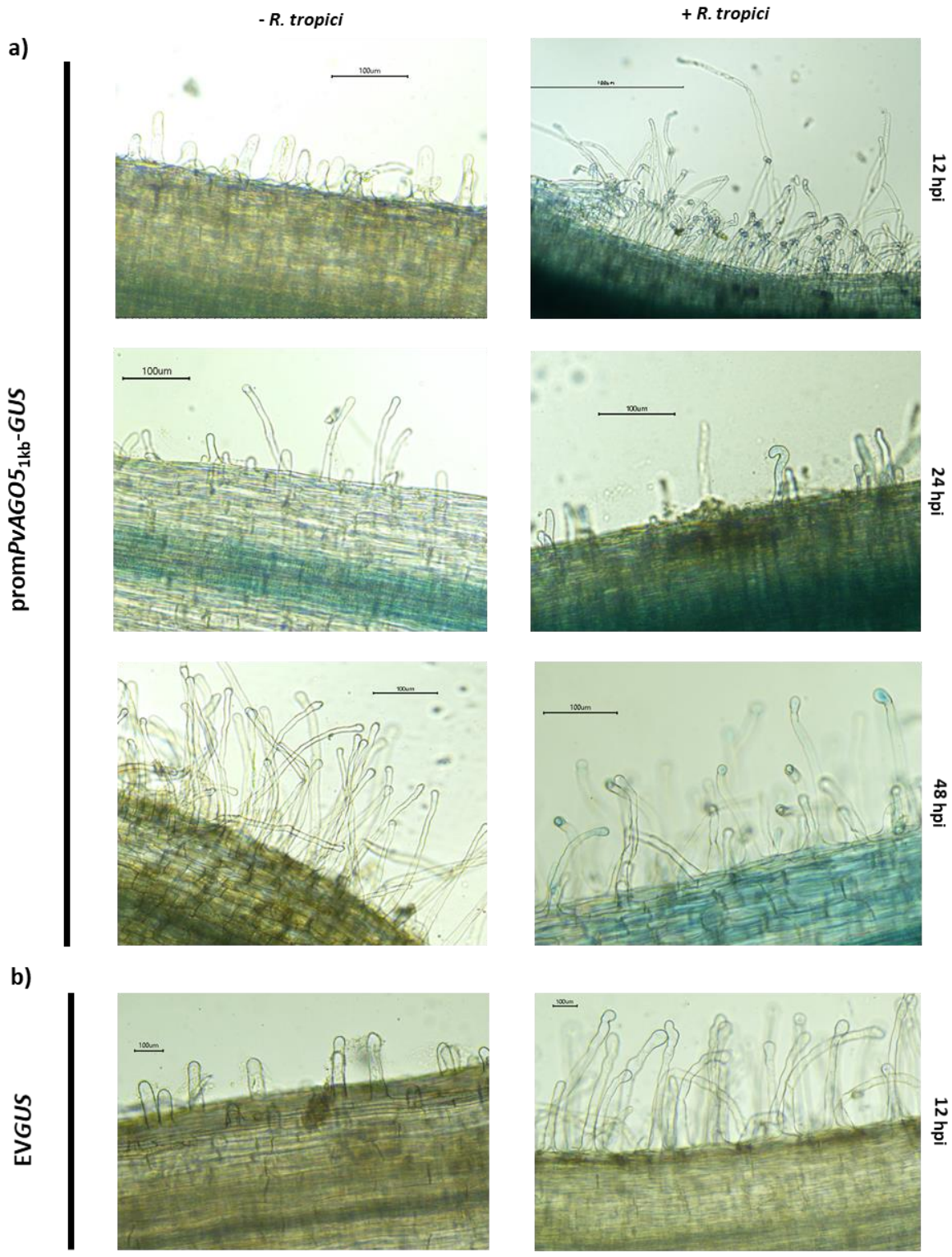

Figura 11. Localización de AGO5 en raíces transgénicas de frijol por actividad de GUS. a) Raíces que llevan la construcción prom*PvAGO5$_{1kb}$-GUS* a diferentes tiempos de inoculación (hpi) con

agua (panel izquiedo) o rizobio (panel derecho). b) Raíces transgénicas control (EV-*GUS*) a las 12 hpi inoculadas con agua inoculadas con agua (izquierda) o rizobio (derecha).

Los nódulos transgénicos de la construcción prom*PvAGO5*$_{1kb}$-*GUS* fueron analizados a los 20 y 25 dpi. La expresión de *AGO5* en los nódulos de 20 dpi se encontró en el meristemo del nódulo (Figura 12 a, panel superior). En las raíces transgénicas la actividad de GUS asociada a la región promotora de *AGO5*, continuó en el cilindro vascular pero se aprecia la forma circular del meristemo del nódulo dentro del córtex de la raíz. Posteriormente a los 25 dpi, AGO5 se localiza en el nódulo maduro, donde se observó en la periferia del nódulo (Figura 12 a, panel central). La actividad de GUS no se observó en los nódulos control de raíces transgenicas con la construcción EV-*GUS* (Figura 12 a, panel inferior).

De los nódulos de 25 dpi se realizaron cortes algunos sin tinción histoquímica y otros teñidos con safranina (Figura 12 b). En nódulos que aún no están bien colonizados, AGO5 se localizó en la zona de fijación (Figura 12 b, panel izquierdo). Cuando las células son infectadas por rizobio, la localización de AGO5 se mantiene en la periferia (Figura 12 b, panel central). En la tinción de safranina, las células infectadas se aprecian en color rosa. Mientras que en la periferia, la tinción morada es producto de la combinación de GUS y safranina (Figura 12 b, panel derecho). En conjunto, estos resultados informan que *AGO5* en respuesta a rizobios se expresa en los pelos radicales, en meristemos de nódulos y en nódulos funcionales, sugiriendo que AGO5 puede participar en la regulación de las etapas asociadas a la infección por rizobio y en la formación del nódulo.

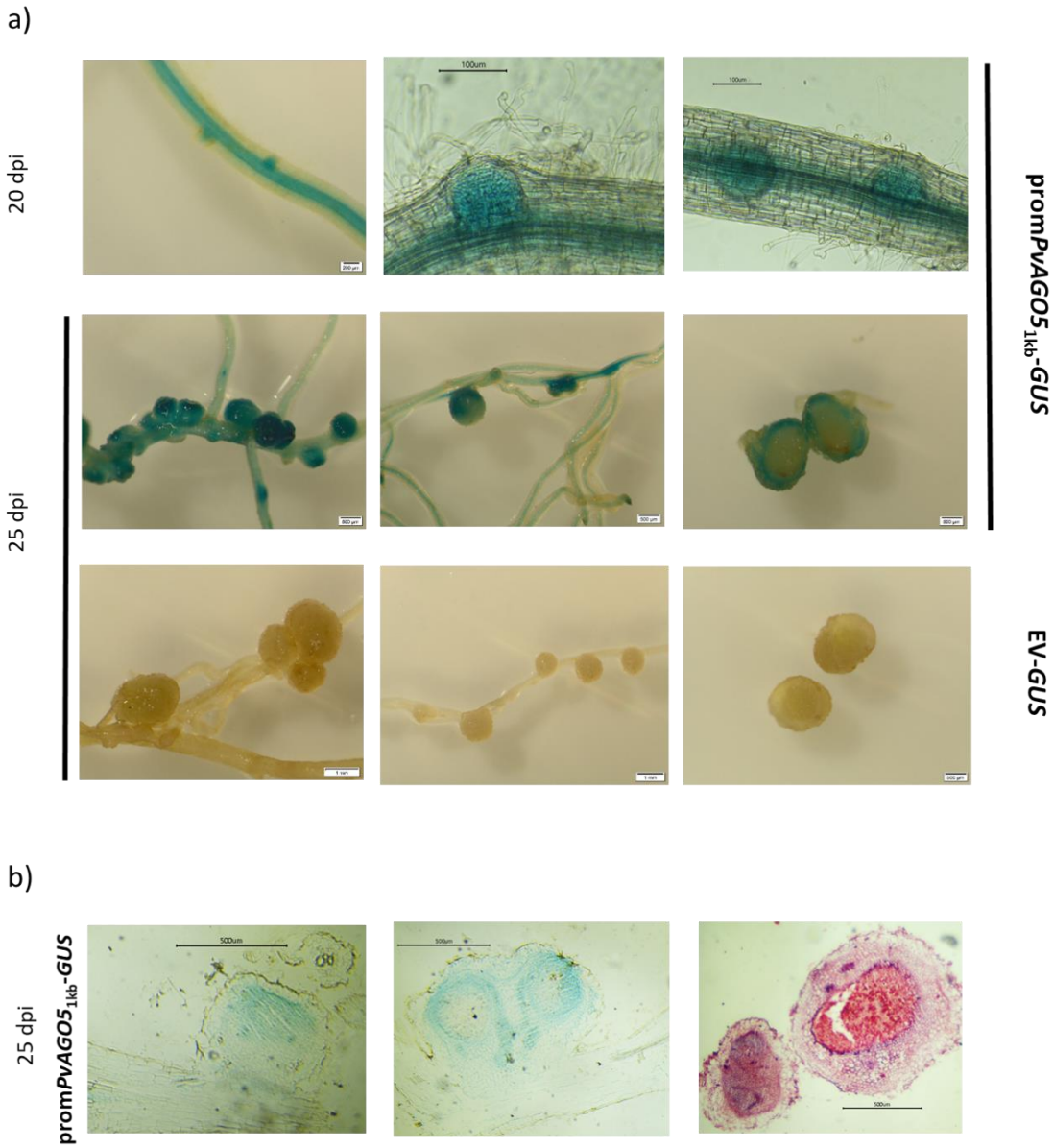

Figura 12. Localización de PvAGO5 en nódulos transgénicos de frijol. a) Nódulos de 20dpi y 25 dpi que llevan la construcción prom*PvAGO5$_{1kb}$-GUS* (panel superior y central) y nódulos de 25 dpi de nódulos control (EV-*GUS*) en el panel inferior. b) Histologia de nódulos de 25 dpi que llevan la construcción prom*PvAGO5$_{1kb}$-GUS*.

5.4. El silenciamiento de *AGO5 afecta la expresión de genes simbióticos.*

Para confirmar la hipótesis de que AGO5 participa en el establecimiento de la simbiosis leguminosa-rizobio, se procedió a silenciar la expresión de AGO5 en raíces de soya y frijol mediante RNAi. Las raíces transgénicas de soya y frijol expresando la construcción *AGO5*-RNAi mostraron una disminución de la expresión de *AGO5* de alrededor del 60 % con respecto a raíces transgénicas expresando el vector vacío (control) (Figura 13).

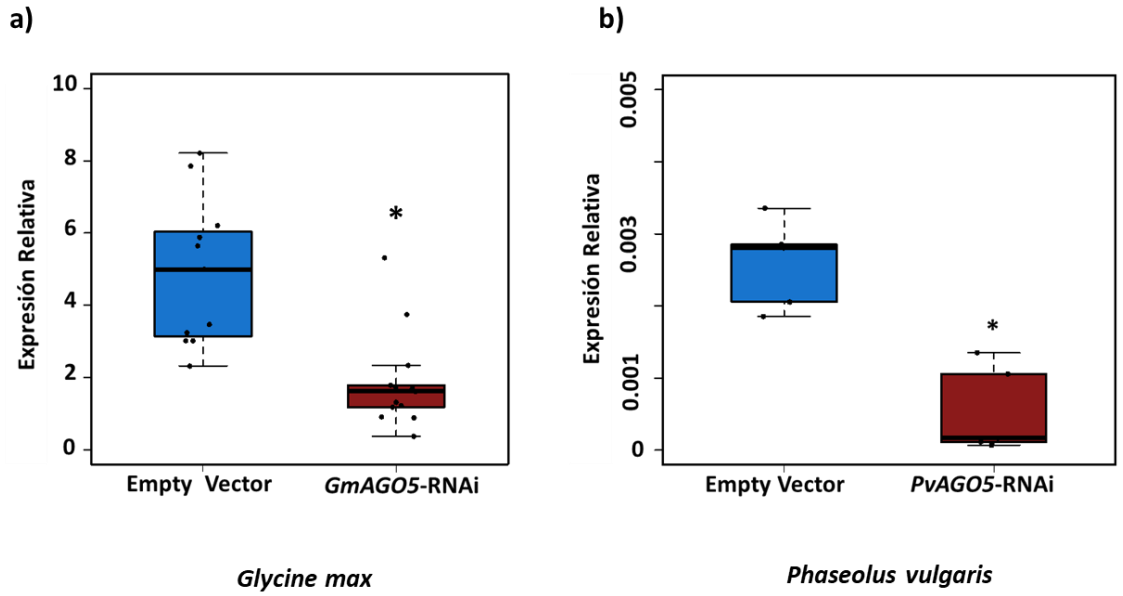

Figura 13. Niveles de expresión de *AGO5* en raíces transgénicas de soya (a) y frijol (*b*) desarrolladas con la construcción de *AGO5-RNAi* e inoculadas con rizobio. Los datos se analizaron mediante un análisis de varianza (ANOVA) seguida de una prueba de Tukey, que mostraron diferencias significativas (*) (P<0.01, n=5).

Una vez corroborada la disminución de la expresión de *AGO5* en las raíces transgénicas de soya y de frijol expresando la construcción de *AGO5*-RNAi, se analizó en raíces transgénicas de frijol el nivel de expresión de los genes simbióticos *PvCYCLOPS, PvNSP2, Pv NIN, PvFLOT2 y PvENOD40,* los cuales participan en el proceso de infección del pelo radical de esta simbiosis. La expresión de estos genes simbióticos fue analizada en la primera hora de interacción con rizobios tiempo en

el que se observó la mayor expresión y acumulación de proteína de AGO5 (Figura 7). Con excepción de *PvCYCLOPS*, el silenciamiento de la expresión de *AGO* disminuyó la expresión de estos genes alrededor de un 50% (Figura 14). Los genes simbióticos analizados disminuyen su expresión durante la primera hora de estar en contacto con su simbionte. Lo cual indica que AGO5 no solo se induce ante la presencia de rizobio, si no que participa en la regulación de los genes simbióticos desde etapas tempranas de la simbiosis leguminosa-rizobio.

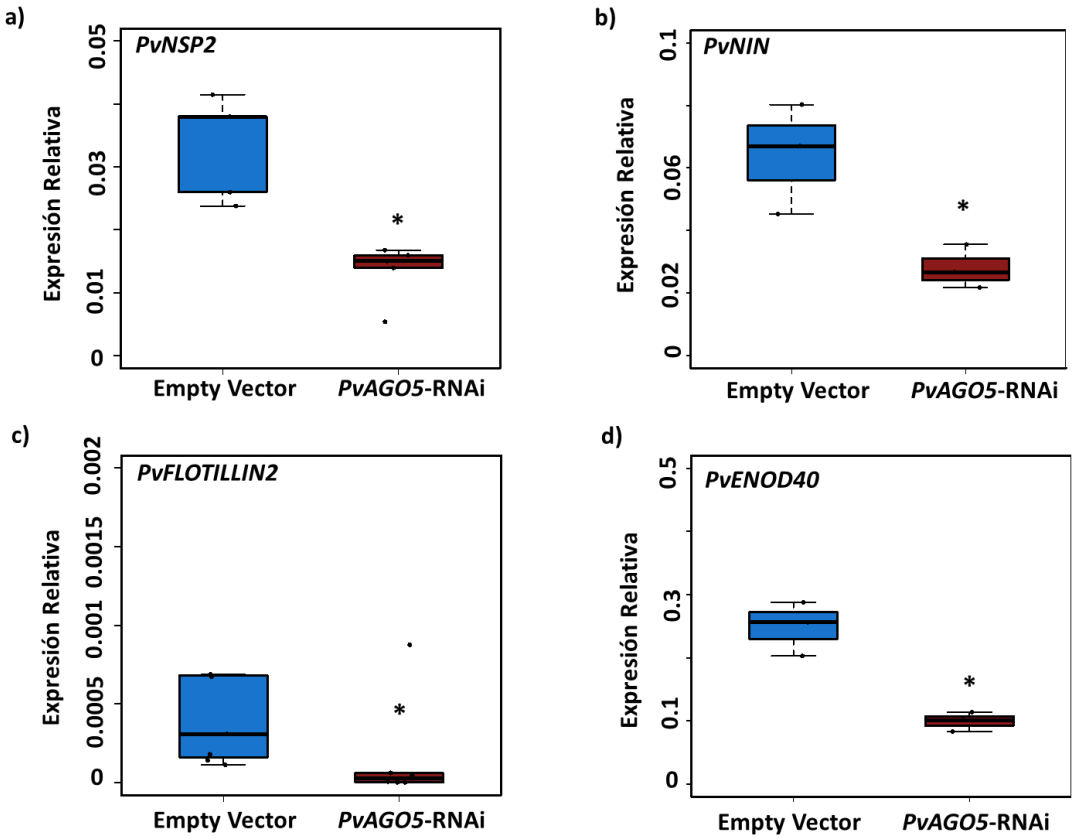

Figura 14. Expresión de genes simbióticos en raíces transgénicas de frijol.que expresan *PvAGO5-RNAi.* Nivel de expresión de (a) *PvNSP2*, (b) *PvNIN*, (c) *PvFLOTILLIN2* y (d) *PvENOD40.* Los datos se analizaron mediante un análisis de varianza (ANOVA) seguida de prueba de Tukey, que mostraron diferencias significativas (*) (P<0.01, n=6).

5.5 AGO5 participa en la deformación del pelo radical y la colonización del rizobio.

Las evidencias obtenidas indican que AGO5 participa en la regulación de la expresión de genes relacionados con el proceso de infección de raíces por rizobios. Por lo tanto, evaluamos el número de pelos radicales deformados por rizobios en raíces transgénicas expresando la construcción *AGO5*-RNAi. Las raíces transgénicas fueron inoculadas con sus respectivos simbiontes (*G.max-B. diazoefficiens, P. vulgaris-R. tropici*) y se observaron con el microscopio a las 48hpi. En las raíces transgénicas de soya no hubo diferencias significativas en el número de pelos deformados por raíz (Figura 15 a, b). Sin embargo, en las raíces con baja expresión de *AGO5* el tipo de deformación más comúnmente observado fueron deformaciones no efectivas “tipo espátula” (Figura 15c, d) (Cerri, *et al*., 2016; Cerri, *et al*., 2017). Se ha reportado que el encorvamiento del pelo radical promueve la cámara de infección para el ingreso del rizobio (Fournier, *et al*., 2015). En este sitio se inicia la formación del hilo de infección, el cual permite a la bacteria colonizar las células del córtex. Por lo que el incremento de deformaciones no efectivas observadas en soya pudo ocasionar mal formaciones de la cámara infectiva. Esto se corroboró en el bajo número de hilos de infección encontrados en las raíces con baja expresión de *AGO5* (Figura 15 e, f).

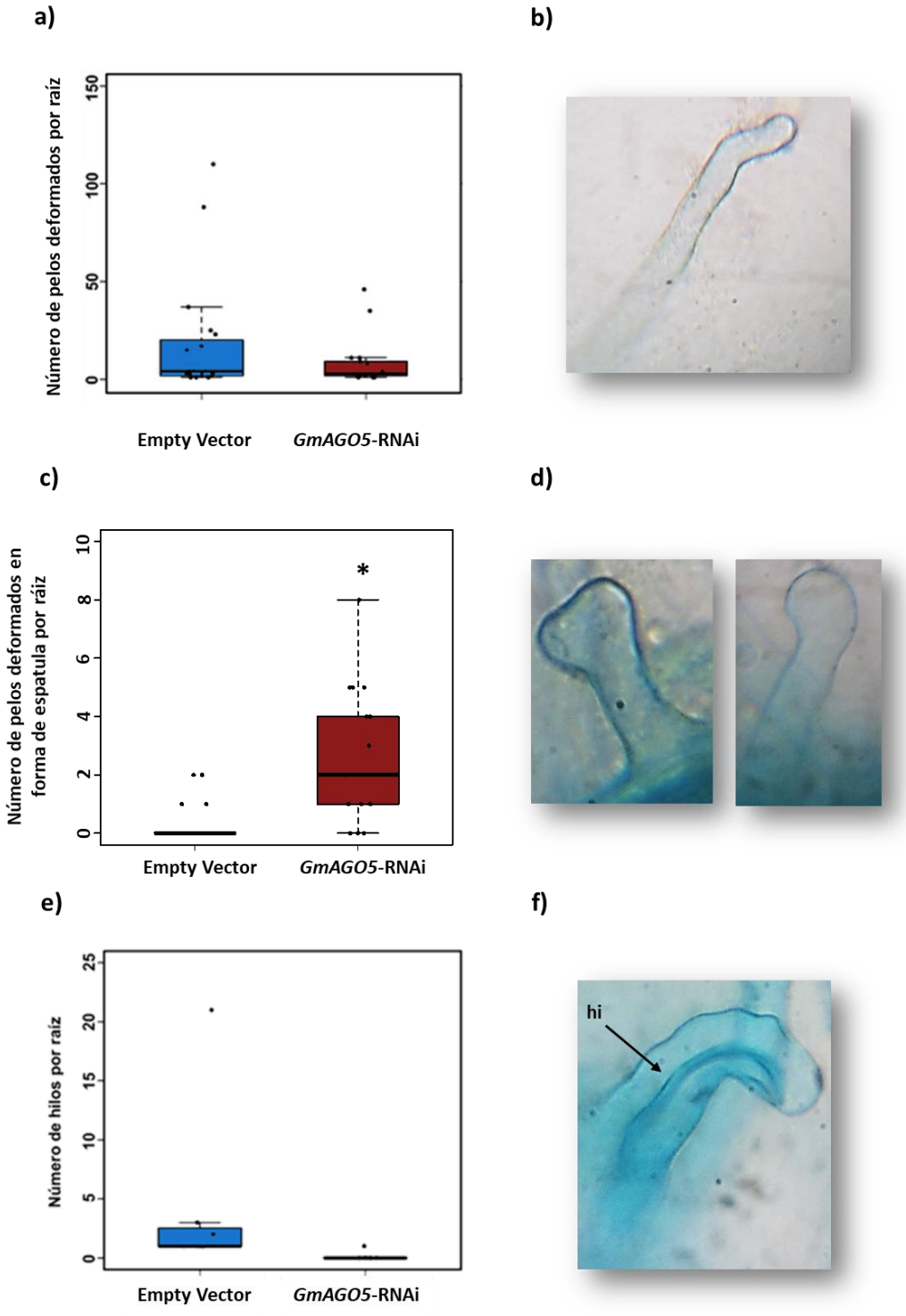

Figura 15. Efectos de la baja expresión de *GmAGO5* en la deformación de pelos radicales de soya. a) Número de pelos deformados por raíz (n=30, P<0.01). b) Deformaciones efectivas en el pelo radical ante la presencia de rizobio. c) Número de deformaciones no efectivas “tipo espátula”.

d) Deformaciones tipo espátula encontradas en *GmAGO5*-RNAi. e) Número de hilos de infección por raíz. f) Hilo de infección (hi) en un pelo radical, se observa el crecimiento desde la cámara de infección.

En lo que respecta a las raíces transgénicas de frijol, el silenciamiento de *AGO5* disminuyó el número de pelos deformados efectivos (Figura 16 a). Estos resultados indican un diferente modo de acción entre ambas leguminosas (sin diferencias en la deformación). Estos eventos morfológicos están relacionados con la baja expresión de genes simbióticos. En conjunto, estos resultados muestran que la baja expresión de *AGO5* en ambas leguminosas afecta la deformación de pelos radicales, indicando que AGO5 participa en el proceso de infección por rizobios.

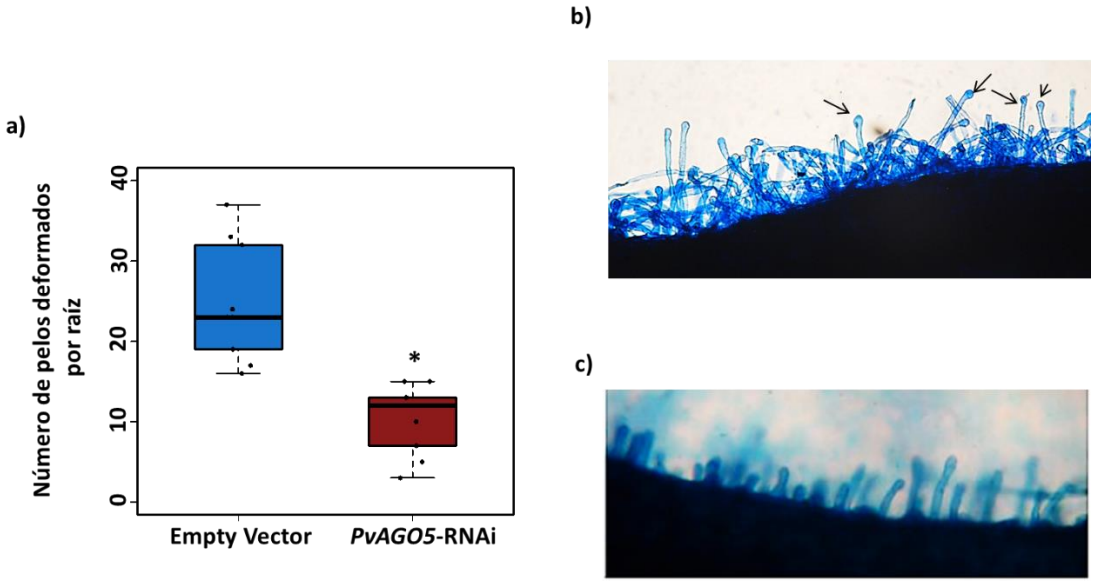

Figura 16. Efecto de la baja expresión de *PvAGO5* en la deformación de pelos radicales de frijol. a) Número de deformaciones efectivas en raíces transgénicas de frijol (Empty vector y *PvAGO5*-RNAi). b) Deformaciones efectivas, las flechas señalan el curling en los pelos radicales. c) Raíces *PvAGO5*-RNAi sin deformaciones efectivas. Los datos se analizaron mediante un análisis de varianza (ANOVA) seguida de una prueba de Tukey, que mostraron diferencias significativas (*) ($P<0.01$, $n=5$).

5.6 AGO5 participa en el desarrollo de nódulos.

Puesto que los datos obtenidos indican que el silenciamiento de *AGO5* afecta el proceso de infección por rizobios, se procedió a evaluar si esta proteína AGO tiene un papel en la formación de los nódulos. Para este fin, se realizó un ensayo de nodulación en raíces transgénicas de soya y de frijol expresando la construcción AGO5-RNAi o el vector vacío (control) a los 30 dias después de inocular con rizobio (dpi). En las dos leguminosas, la baja expresión de *AGO5* se correlaciona con la disminución del número de nódulos por planta (Figura 17 a, b). Así mismo, en ambas leguminosas el tamaño de los nódulos fue más pequeño, en comparación con los nódulos de raíces control. Por otra parte, la disección en fresco mostró en los nódulos control el color rosa dado por la leghemoglobina, mientras que en los nódulos con baja expresión de *AGO5* fueron color rosa pálido o en su mayoría blancos, indicando que estos nódulos poseen una baja cantidad de leghemoglobina, lo cual nos lleva a sugerir que estos nódulos no fijan eficientemente nitrógeno y que puede haber pocos bacteroides dentro del nódulo (Figura 17 c, d).

La hipótesis de la poca colonización de los bacteroides en el nódulo se corroboró mediante el análisis histológico de los nódulos *AGO5*-RNAi. En ambas leguminosas, los cortes de nódulos control mostraron un tejido cortical bien definido y una gran cantidad de células infectadas por rizobio (Figura 18 a, b). En contraste, los nódulos *AGO5*-RNAi mostraron pocas células infectadas con un tejido cortical bien definido (Figura 18 c). La diferencia en contenido de células infectadas por rizobio se puede atribuir a la baja expresión de *AGO5* y no al tamaño del nódulo, como se puede apreciar el los nódulos de tamaño similar entre el control y *AGO5*-RNAi de frijol (Figura 18d). En conjunto, estos datos indican que AGO5 también está implicada en el desarrollo de los nódulos y en la infección por rizobios de sus células.

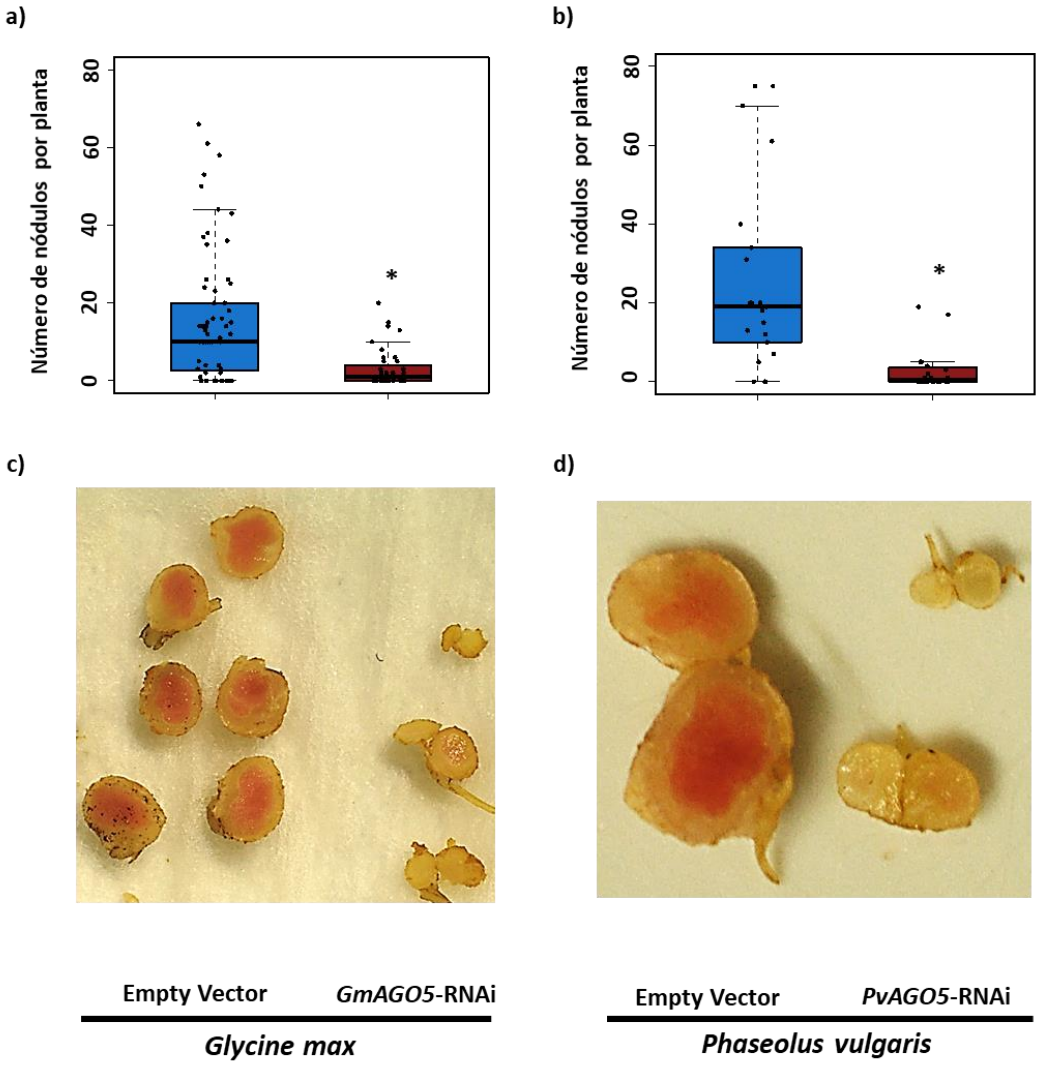

Figura 17. La baja expresión de *AGO5* afecta el proceso de nodulación. Número de nódulos en soya (a) y frijol (b). Tamaño de los nódulos transgénicos *AGO5*-RNAi de soya (c) y de frijol (d). Los datos se analizaron mediante un análisis de varianza (ANOVA) seguida de una prueba de Tukey, que mostraron diferencias significativas (*) ($P<0.01$, $n=30$).

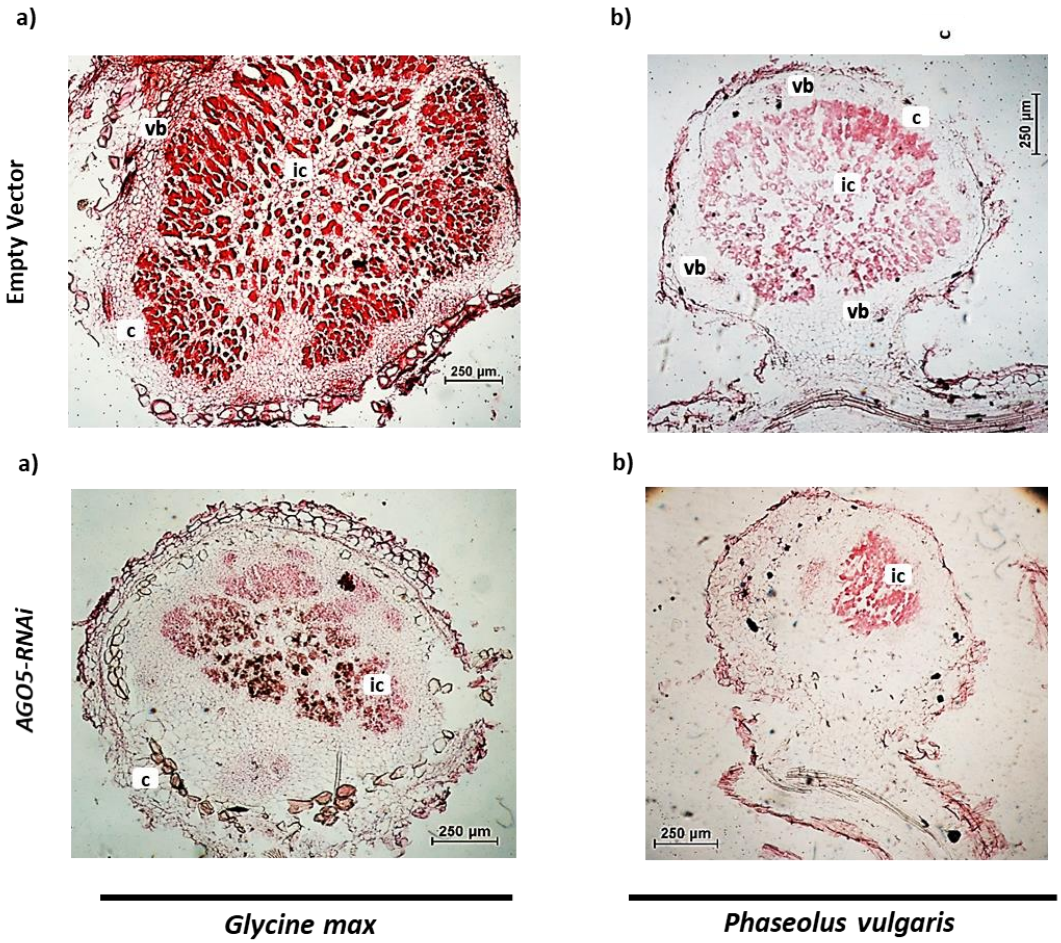

Figura 18. Histología de nódulos con baja expresión de *AGO5.* Nódulos transgénicos control (Vector vacío) de soya y de frijol con células infectadas con bacteroides (a, b). Nódulos *AGO5*-RNAi transgénicos de soya (c) y frijol (d) con pocas células infectadas. C: córtex; ic: células infectadas; vb: haces vasculares.

6. DISCUSIÓN.

Los mecanismos de señalización en la simbiosis leguminosa-rizobio se han estudiado desde hace más de dos décadas. Hasta el momento se han descubierto casi 200 genes involucrados en el proceso de la FSN (Sonali, *et al*., 2020). Dentro de los componentes estudiados que regulan la simbiosis, se encuentran los miRNAs, los cuales están involucrados en la regulación del proceso de infección y el desarrollo del nódulo (Combier, *et al*., 2006; Boualem, *et al*., 2008; Bazin, *et al*., 2012). Sin embargo, no se ha hecho un estudio sobre las proteínas Argonauta, las cuales forman junto con el miRNA el complejo RISC. Recientemente, la importancia de las proteínas AGO durante la simbiosis ha comenzado a ser objeto de interés en el campo de la FSN, así como su participación en la maquinaria postranscripcional del proceso de infección del rizobio (Roy, *et al,* 2019; Hoang, *et al.,* 2020). En base a lo anterior, se ha hipotetizado sobre el posible papel que pueden tener estas proteínas a lo largo de cada una de las etapas de la simbosis entre leguminosas y rizobios (Valdés-López, *et al.,* 2019, Anexo ll). También se ha reportado que en soya y frijol la proteína AGO5 está involucrada en el proceso simbiótico, desde las etapas tempranas hasta los eventos tardíos, como el desarrollo del nódulo (Hoang, *et al*., 2020).

Las proteínas AGO están implicadas en diferentes procesos de adaptación de las plantas ante cambios ambientales e interacciones con microorganismos (Fang & Qi, 2016). En *A. thaliana*, AGO5 se induce ante la presencia del virus de la papa (Brosseau & Moffett, 2015) lo cual indica su actividad durante la interacción planta-pátogeno. Los pátogenos y simbiontes poseen diferentes mecanismos que influyen en el crecimiento de las plantas, llegando a converger en el tipo de percepción (Desbrosses & Stougaard, 2011). En este trabajo, se muestran evidencias de que la presencia de rizobio induce la expresión y acumulación de AGO5, desde etapas muy tempranas de la interacción simbiótica entre leguminosas y rizobios.

La comunicación para el establecimiento de las simbiosis entre la leguminosas y rizobio inicia con el reconocimiento de los FNs, lo cual desencadena la señalización

necesaria para el establecimiento de la simbiosis (Limpens, *et al*., 2003; Madsen, *et al*., 2003; Radutoiu, *et al*., 2003). Esta señalización involucra la fosforilación de diferentes factores de transcripción que regulan la expresión de genes simbióticos, que son requeridos para la infección del rizobio (Messinese, *et al*., 2007, Yano, *et al*., 2008; Oldroyd, *et al*., 2011). Entre estos factores de transcripción se encuentran los NSP1 y NSP2, los cuales activan la expresión de *NIN*, que es un regulador clave para los genes involucrados en el proceso de infección, colonización y formación de los hilos de infección (HI) (Marsh, *et al*., 2007; Soyano, *et al*., 2013; Liu, *et al*., 2019). Debido aque se observó la inducción de la expresión de *AGO5* desde etapas tempranas (primeras tres horas), se analizó como afectaba la baja expresión de este gen en la expresión de genes simbióticos. Las raíces transgénicas *PvAGO5*-RNAi tuvieron una disminución del 50% en la expresión de los genes simbióticos *PvNSP2, PvNIN, PvFLOT2* y *PvENOD40*. Se sabe que la infección por rizobio es sensible a defectos en la señalización, lo que puede resultar en la supresión parcial de algunos genes (Caopen, *et al*., 2005; Limpens, *et al*., 2005). Esta alteración en la expresión de los genes simbióticos se reflejó en eventos morfológicos asociados a la infección del rizobio. En las raíces transgénicas de frijol, el número de pelos radicales deformados disminuyó significativamente. Esto puede atribuirse al decremento en la expresión de genes simbióticos importantes como *NSP2* y *FLOT2.* A través de el estudio de mutaciones en la via de señalización de la simbiosis, se ha visto que pueden ocurrir eventos de infección defectuosos, que van desde el retraso en la aparición de los hilos de infección (mutantes *Mtipd3*), la reducción en la deformación de pelos radicales (mutantes *Mtnsp2*), o el incremento en deformaciones “tipo espátula” (Oldroyd & Long, 2003; Haney & Long, 2010; Horvath, *et al*., 2011; Cerri, 2016). Este tipo de deformación se presenta en pelos que inician su encorvamiento pero son incapaces de atrapar a la bacteria y no pueden desarrollar una cámara de infección que permita el ingreso del rizobio (Cerri, 2016). Las deformaciones “tipo espatula” se han descrito en leguminosas mutantes *ern1* (Cerri, *et al*., 2016; Cerri, *et al*., 2017). En estas mutantes, este tipo de deformación va acompañada de una falta de HI, tal y como lo observamos en nuestro estudio.

La evidencia presentada en este estudio indica que la expresión de *AGO5* es inducida por el rizobio desde etapas tempranas, lo cual tiene sustento en la localización de AGO5 en la punta de los pelos radicales. El análisis de elementos *cis* en la región promotora de *AGO5* de soya y frijol permitió identificar potenciales elementos *cis* que pudieran controlar su expresión en cada una de las etapas de la simbiosis leguminosa-rizobio. Por ejemplo, se encontró el elemento *cis* GT1-GMSCAM4, el cual es importante par la inducción del gen *SCaM-4*, que es una isoforma de una calmodulina de unión a calcio que se induce ante la interacción de soya con un patógeno (Park, *et al*., 2004).

Los resultados obtenidos en este trabajo, también indican que AGO5 participa en el desarrollo del nódulo. El silenciamiento de *AGO5* disminuye la expresión de los genes requeridos para el desarrollo del nódulo tales como: *NIN, NSP2* y *ENOD40* los cuales regulan la formación del nódulo (Wang, *et al*., 2015). Esta disminución en la expresión de los genes simbióticos repercutió en el desarrollo de los nódulos de soya y frijol, los cuales fueron pequeños y de color blanco. Este fenotipo se ha observado en plantas cuyos nódulos presenta una capacidad de fijación reducida (Roy, *et al*., 2019) o presentan problemas en la infección por rizobio (Xie, *et al*., 2012, Liu, *et al*., 2019). Adicionalmente, la localización de AGO5 en el primordio nodular y en el nódulo maduro sostiene el papel de esta proteína durante el desarrollo del nódulo. En la región promotora de *AGO5* analizada se pudieron identificar 4 elementos *cis* (NODCON2GM, OSE2ROOTNODULE) cuya secuencia es un elemento específico de órgano (OSE). Particularmente en soya se ha observado que ésta es esencial para la leghemoblobina N23 y otras nodulinas que se expresan en las células infectadas del nódulo (Stougaard, *et al*., 1990). El promotor de *AGO5* posee elementos *cis* que responden desde etapas tempranas y otros involucrados en el desarrollo del nódulo, sin embargo, la identificación de los elementos *cis* que controlan la expresión de AGO5 en esta simbiosis tienen que ser estudiados a detalle.

Las evidencias mostradas en este trabajo indican que AGO5 induce su expresión debido a la presencia de rizobio. Esta proteína participa en la regulación de los

genes simbióticos que involucran la deformación del pelo radical, la infección del rizobio y la organogénesis del nódulo (Figura 19).

La familia de las proteínas AGO se ha expandido durante la evolución y diversificado en las plantas (Singh, *et al*., 2015; Fang & Qi, *et al*., 2016). AGO5 es una proteína que además de estar involucrada en la defensa de la planta en las interacciones con algún patógeno (Takeda, *et al*., 2008; Vaucheret, 2008) también está cumpliendo un papel en las interacciones simbióticas. Esto nos indica la importancia de las proteínas AGO en las redes de regulacion transcripcional, post-transcripcional y post-traduccional, ya que dependiendo de la interacción que tenga la planta con un patógeno o simbionte, será la respuesta a esa interacción especifica.

La gran cantidad de estudios que se han realizado sobre los miRNAs que participan en la simbiosis (Hoang, *et al*., 2020) también es un claro ejemplo de que las proteínas AGO deben tener un papel importante en la regulación de este proceso. Existen miRNAs (e.g. miR172c, miR171c, miR397) que participan en la deformación del pelo radical y la infección del rizobio (Valdés-López*, et al*., 2019a, Anexo ll). Por lo que es de esperar, que las proteínas AGO se encuentren presentes desde el inicio de esta simbiosis y que mantengan su participación, como hemos evidenciado en nuestro trabajo, hasta el desarrollo del nódulo. E inclusive, existe la probabilidad de que sea necesaria la participación de mas de una AGO, como se ha visto en otros procesos del desarrollo de las plantas, donde AGO10 y AGO1 participan en la formación del meristemo apical (Zhu, *et al*., 2011; Ji, *et al*., 2011). Además, el análisis filogenético que realizamos muestra que las AGO se han multiplicado en las leguminosas, posiblemente como respuesta al proceso simbiótico. Sin embargo, son necesarios mas estudios que involucren a esta familia de proteínas durante el establecimiento de este proceso simbiótico.

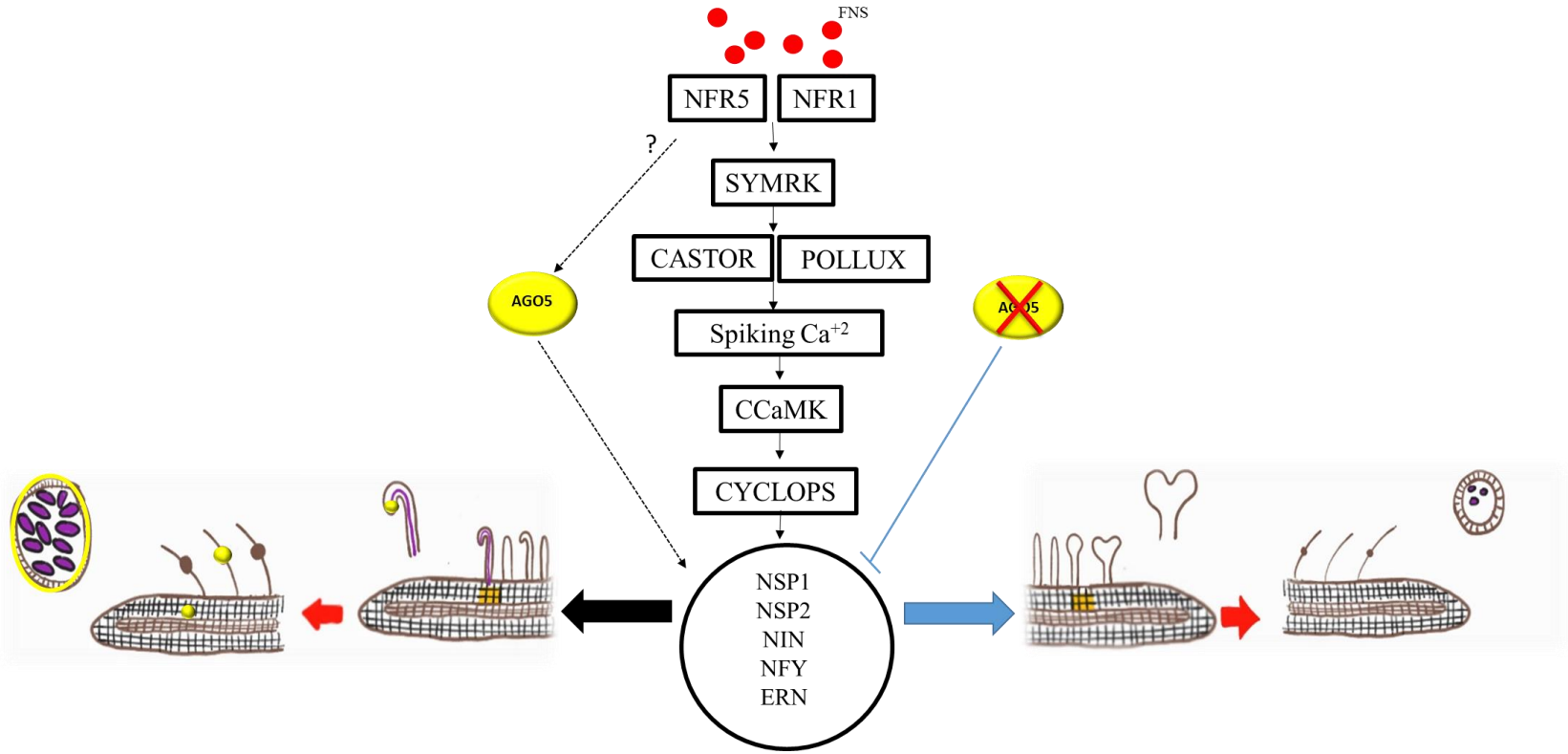

Figura 19. Modelo del papel de AGO5 durante la simbiosis leguminosa-rizobio. A la izquierda AGO5 es inducida por la presencia de rizobio, lo cual afecta la expresión de los genes simbióticos, probablemente río abajo de CYCLOPS. AGO5 participa en la deformación del pelo radical y se localiza (amarillo) en la punta de los pelos deformados, asi como en los meristemos nodulares y en la periferia del nódulo maduro. A la derecha, el silenciamiento de *AGO5* disminuye la expresión de genes simbióticos, ocasionando problemas en la deformación del pelo radical y en la infección. Los nódulos se reducen en número y tamaño, así como en el contenido de células infectadas por bacteroides.

7. CONCLUSIONES.

Las proteínas AGO forman parte de complejo RISC, el cual a través de su interacción con sRNAs regulan diferentes etapas del proceso simbiótico leguminosa-rizobio. Se han realizado diversos estudios sobre como algunos miRNAs intervienen en este proceso simbiótico pero se ha dejado a un lado como la proteína AGO puede jugar un papel en la regulación de dicho proceso, por lo que este trabajo abordó el papel de AGO5 durante la interacción simbiótica.

Las evidencias muestran que esta proteína interviene desde etapas muy tempranas de la interacción leguminosa-rizobio. Su silenciamiento afectó la expresión de genes simbióticos y los procesos de infección del rizobio. La proteína AGO5 se localiza en la punta del pelo radical y posteriormente en el primordio nodular, donde también interviene en el desarrollo del nódulo. Este estudio, es uno de los primeros trabajos en los que se reporta el papel de una proteína AGO durante la simbiosis leguminosa-rizobio.

8. PERSPECTIVAS

La proteína AGO5 interviene a lo largo del proceso simbiótico entre plantas leguminosas y rizobios. Desde las primeras horas en que detecta el rizobio hasta la formación del primordio nodular. Uno de los eventos que se ven modificados durante el silenciamiento de *AGO5* es la expresión de los genes simbióticos, pero desconocemos como se lleva a cabo esta regulación. El análisis de los sRNAs que se unen a AGO5 puede develar información más precisa de cómo esta proteína regula este proceso simbiótico e inclusive puede permitirnos esclarecer en que parte de la vía simbiótica es donde empieza su papel regulador.

Otra hipótesis es que si AGO5 es inducida por la presencia de rizobios, puede ser que responda ante la presencia de un patógeno. Lo cual nos indicaría que AGO5 juega un papel no solo en la interacción simbiótica sino también en la regulación de la respuesta de las leguminosas ante la interacción con diversos microorganismos. Ante este escenario podría AGO5 ser un componente que ayude a la leguminosa a reconocer entre un simbionte y el ataque de un patógeno.

Las proteínas AGO han sido estudiadas sobre todo en la planta modelo *A. thaliana* y se ha reportado que juegan un papel importante durante el desarrollo de la planta, incluso entre proteínas AGO del mismo clado (i. e AGO1 y AGO10) tienen funciones redundantes entre ellas. Sabemos que AGO5 está participando en el proceso simbiótico de la interacción de leguminosa-rizobio y probablemente existan otras AGO involucradas en este proceso, por lo que el estudio detallado de la participación de estas proteínas en el proceso simbiótico, así como su posible interacción y/o redundancia entre los procesos que controlan es un tema basto para seguir investigando.

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10. ANEXOS

Los siguientes anexos corresponden a los artículos desarrollados durante mis estudios de doctorado citados en esta tesis.

Anexo I

Reyero-Saavedra, M. R., Qiao, Z., Sánchez-Correa, M. S., Díaz-Pineda, M. E., Reyes, J. L., Covarrubias, A. A., Libault, M., et al., 2017. Gene Silencing of Argonaute5 negatively affects the establishment of the legume-rhizobia symbiosis. Genes. 8: 352.

Anexo ll

Valdés-López, O., Formey, D., Isidra-Arellano, M. C., Reyero Saavedra, M. R., Fernandez-Gobel, T. F., Sánchez-Correa, M. S. 2019. Argonaute Proteins: Why are they so important for the Legume-Rhizobia Symbiosis?. Frontiers in Plant Science. 10: 1177.

Anexo lll

Valdés-López, O., Jayaraman, D., Maeda, J., Delaux, P.-M., Venkateshwaran, M., Isidra-Arellano, M. C., Reyero-Saavedra, M. R., et al. 2019. A novel positive regulator of the early stages of root nodule symbiosis identified by phosphoproteomics. Plant Cell Physiol. 60 (3): 575-586.

genes

MDPI

Article

Gene Silencing of *Argonaute5* Negatively Affects the Establishment of the Legume-Rhizobia Symbiosis

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Abstract: The establishment of the symbiosis between legumes and nitrogen-fixing rhizobia is finely regulated at the transcriptional, posttranscriptional and posttranslational levels. Argonaute5 (AGO5), a protein involved in RNA silencing, can bind both viral RNAs and microRNAs to control plant-microbe interactions and plant physiology. For instance, AGO5 regulates the systemic resistance of Arabidopsis against Potato Virus X as well as the pigmentation of soybean (*Glycine max*) seeds. Here, we show that AGO5 is also playing a central role in legume nodulation based on its preferential expression in common bean (*Phaseolus vulgaris*) and soybean roots and nodules. We also report that the expression of AGO5 is induced after 1 h of inoculation with rhizobia. Down-regulation of *AGO5* gene in *P. vulgaris* and *G. max* causes diminished root hair curling, reduces nodule formation and interferes with the induction of three critical symbiotic genes: Nuclear Factor Y-B (*NF-YB*), Nodule Inception (*NIN*) and Flotillin2 (*FLOT2*). Our findings provide evidence that the common bean and soybean *AGO5* genes play an essential role in the establishment of the symbiosis with rhizobia.

Keywords: common bean; soybean; Argonaute5; legume-rhizobia symbiosis

1. Introduction

Legumes can establish symbiosis with nitrogen-fixing bacteria (rhizobia). Through this symbiosis, atmospheric nitrogen is fixed before being assimilated (i.e., amino acids) by the plant [1]. Hence, the symbiosis between legumes and rhizobia has a considerable relevance at the ecological level. In fact, it has been estimated that the legume-rhizobia symbiosis fixes 60 million metric tons of nitrogen worldwide, and reduces the use of synthetic fertilizers [2].

To establish this symbiosis, a molecular dialog between rhizobia and legume partners is required [3,4]. This dialog begins with the detection by compatible rhizobia of legume-produced flavonoids and isoflavonoids [3,4]. In response, the rhizobia synthesize and exude lipo-chitooligosaccharides (LCOs), known as Nod Factors (NFs). The legume-host perceives NFs via LysM-domain receptor kinases Nod Factor Receptor1 and 5 (NFR1 and NFR5), both located at the legume root hair plasma membrane. Upon NFs perception, the transcription and phosphorylation

of several symbiosis-related genes and proteins is activated, respectively [5]. These molecular responses are required for subsequent steps of rhizobial infection and the formation of a new organ, the nodule [4,5]. For instance, rhizobia colonize legume roots through the infection of the epidermal root hairs [6]. This colonization process begins with the attachment of rhizobia to a growing root hair tip, which induces a continuous reorientation of the tip growth, eventually leading to root hair deformation or curling [6]. In the center of this curl, an infection chamber is formed, where rhizobia are entrapped and multiply to form a micro-colony [7]. Upon rhizobia entrapment, an infection thread is formed, initiated at the location of the infection chamber and elongating to reach the nodule primordium, a meristem initiated via cell division of root cortical cells [8]. Rhizobia within the infection thread are then released into the nodule primordium cells and differentiate into bacteroids that are now able to fix nitrogen within the nodule [5].

Although the infection of root hair cells by rhizobia and the development of the nodule are biological processes controlled by two independent genetic programs, they are finely coordinated by a set of symbiotic genes [3,4]. Among these genes, *NFR5* and *NFR1*, along with *SYMRK/DMI2/NORK* (in *Lotus japonicus*, *Medicago truncatula* and *Medicago sativa*, respectively), which encode a leucine-rich repeat (LRR) receptor like kinase, are required for the perception and transduction of the NFs signal [4,9]. As a first response to NFs perception, legumes activate the expression of the potassium-permeable channel *DMI1*, calcium channels of the *CNGC15* family, the calcium pump *MCA8*, and nucleoporins (*NUP85*, *NUP133*, and *NENA*), as well as the expression of the mevalonate biosynthesis pathway. These genes are required to generate rapid oscillations in the nuclear and perinuclear calcium concentrations known as calcium spiking [10–17]. To decode the calcium spiking, a calcium and calmodulin-dependent protein kinase (CCaMK) is activated, which phosphorylates the transcription factor CYCLOPS [18,19]. Acting downstream, transcription factors such as Nodulation-signalling pathway1 (NSP1 and NSP2), Nodule inception protein (NIN), Ethylene response factor required for nodulation1 (ERN1), and Nuclear factor YA-1 and YB-1 (NF-YA1 and NF-YB1), are activated. The coordinated action of all these transcription factors is essential to activate the expression of different genes required for the infection of the root hair cell by rhizobia [3,4].

Recent evidence indicates that the expression of several symbiotic genes, in both rhizobia and legumes, is regulated at the epigenetic level [20]. For instance, in the legume model *M. truncatula*, demethylation of genomic DNA by DEMETER (DME) regulates the expression of genes encoding Nodule-specific Cysteine-Rich (*NCR*), Calmodulin-like, and leghemoglobin proteins, which are all required for both rhizobia differentiation and nodule development [21,22]. Similarly, the methylation pattern of the rhizobial genome affects their ability to form nodules in legumes [20]. For instance, overexpression of the DNA methyltransferase *CcrM* in *Mesorhizobium loti* leads to the deregulation of the methylation profile of the microbial genomic DNA (gDNA) leading to a delay in the development of *L. japonicus* nodules [23].

Argonaute (AGO) proteins bind small RNAs to form RNA-induced silencing complexes (RISC) involved in transcriptional and posttranscriptional gene silencing. *Arabidopsis thaliana* genome encodes ten AGO proteins (i.e., AGO1 to AGO10 [24]). Comparative genomic studies revealed the differential evolution of the *AGO* family in various flowering plants upon gene duplication and functional divergence. For instance, soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) encode 23 and 14 AGO proteins, respectively. It has been hypothesized that this duplication led to new, diverged or specific biological functions of the AGO proteins [25]. To date, AGO proteins have been involved in different developmental process and in the adaptation of plants to the changing environment, including their interaction with microbes [24]. For instance, as supported by the role of different microRNAs as major regulators of the nodule process, AGO1 has been indirectly associated with the regulation of the symbiosis between legumes and rhizobia [26]. Other AGO proteins have been involved in the control of the reproductive stage; for instance, there is evidence supporting the role of AGO5 and AGO9 in gametogenesis and in the restriction of the number of megaspore mother cells, respectively [27–29].

Although the genetic control underlying the establishment of the symbiosis between legumes and rhizobia has been extensively studied over the past two decades, large-scale analyses (e.g., transcriptomics and phosphoproteomics) from rhizobia-inoculated or NFs-treated roots from different legumes have revealed the existence of several potential new regulators of the symbiosis between legumes and rhizobia [30–32]. However, most of these genes has not been functionally characterized.

Here, we report the functional characterization of one of these potential new regulator of this symbiosis, AGO5, in common bean and soybean, two major crop legumes. Upon mining of the common bean and soybean transcriptional databases [33–35], we found *AGO5* preferentially expressed in roots and nodules. Further experimental validation revealed that *AGO5* is induced in response to rhizobia. To demonstrate the role of AGO5 during nodulation, we applied an RNAi strategy to down-regulate its expression. Upon silencing of *AGO5* genes in *P. vulgaris* and *G. max*, we observed a defect in nodule formation and in the induction of three critical symbiotic genes: *NF-YB*, *NIN* and Flotillin2 (*FLOT2*). Our findings show that AGO5 might play an essential role in the establishment of the symbiosis between rhizobia and legumes.

2. Material and Methods

2.1. Plant Material

Common bean (*P. vulgaris* L. cv Negro Jamapa) and soybean (*G. max* L. (Merrill) Williams 82) seeds were kindly provided by Dr. Georgina Hernandez from the Center for Genomics Science, UNAM, at Cuernavaca, Morelos, Mexico, and by Dr. Gary Stacey from the University of Missouri at Columbia, Missouri, USA. Seeds were surfaced sterilized by soaking in 70% ethanol for 1 min, followed by treatment for 10 min with 10% bleach. Seeds were subsequently washed ten-times in sterile water. Sterilized common bean seeds were germinated for two days in Petri dishes containing sterile wet germination paper under dark conditions at 25 °C. After three days of germination, common bean seedlings were transferred into 25 cm × 25 cm Petri dishes containing nitrogen-free Fähraeus medium [36] or into pots containing wet agrolite. Sterilized soybean seeds were germinated for three days in 25 cm × 25 cm Petri dishes containing nitrogen-free Fähraeus medium at 25 °C and in dark conditions. Soybean seedlings were kept under these conditions for further analyses or transferred into pots containing wet agrolite.

2.2. Bacterial Strains and Culture Conditions

The empty vector pTDT-DC-RNAi and the hairpin RNA interference (RNAi) construct against common bean and soybean *AGO5* (see below for details) were propagated in *Escherichia coli* DB 3.1 and DH5α cells, respectively. *E. coli* bacterial cells were handled using standard procedures.

Agrobacterium rhizogenes K599 strain was used to induce transgenic roots in common bean and soybean plants (see below for details). *A. rhizogenes* cells were grown on 5 mg/L peptone/3 mg/L yeast extract (PY) plates for two days at 30 °C. 100 µg/mL spectinomycin was added to select for the presence of plasmid vectors.

Rhizobium tropici CIAT899 and *Bradyrhizobium diazoefficiens USDA110* (reclassified from *Bradyrhizobium japonicum*) strains were used to inoculate common bean and soybean plants, respectively. *R. tropici* cells were grown on PY plates supplemented with 20 µg/mL nalidixic acid for two days at 30 °C. *B. diazoefficiens* cells were grown on YEM (0.4 g/L yeast extract, 10 g/L mannitol, 0.2 g/L $MgSO_4$, 0.5 g/L $KHPO_4$, 0.1g/L NaCl, pH 7.0) plates for four days at 30 °C.

2.3. AGO5 Down-Regulation by RNA Interference

A 150 bp 3'UTR fragment was used to generate a hairpin RNAi against *AGO5*. The amplified fragment was then cloned into the pENTR-D-TOPO (Thermo Fisher Scientific, Waltham, MA, USA) vector and verified by sequencing. The resulting pENTR-*AGO5*-RNAi plasmid was recombined into the pTDT-DC-RNAi binary vector containing the constitutively expressed fluorescent Tandem

Dimer Tomato (*tdTomato*) reporter gene [37]. The correct orientation was verified by Polymerase Chain Reaction (PCR) using the primers WRKY Intron-fwd and *AGO5*-rev (for oligonucleotide sequences see Table S1). *A. rhizogenes* K599 was transformed with this RNAi vector or with the control empty vector (pTDT-DC-RNAi). *A. rhizogenes*-mediated transformation of common bean and soybean plants was performed according to [38,39], respectively. The transgenic roots were selected upon observation of TDT fluorescence with an epifluorescence stereomicroscope (SZX10, Olympus, Center Valley, PA, USA) equipped with an Olympus UC50 camera (Olympus).

2.4. Treatments

Three day-old soybean and common bean seedlings were transferred into nitrogen-free Fähraeus plates. Two days after transplanting, seedlings were inoculated with *R. tropici* CIAT899 (common bean symbiont) or *B. diazoefficiens* USDA110 (soybean symbiont). Inoculated seedlings were kept under dark conditions at room temperature (RT). At 1, 3, 6, 12, 24 and 48 h post inoculation, roots were harvested in liquid nitrogen and stored at −80 °C until used for transcriptional analyses. Additionally, leaves and roots from three-week-old plants as well as 25 day-old nodules were harvested in liquid nitrogen and stored at −80 °C until use. Three biological replicates were included.

Composite plants (plants with transformed root system and untransformed shoot system), expressing the construct *AGO5*-RNAi or control vector were transferred into 25 × 25 cm Petri dishes containing nitrogen-free Fähraeus medium. After four days, transgenic roots were inoculated with *R. tropici* (common bean composite plants) or *B. japonicum* (soybean composite plants). One hour after inoculation, the tdTomato fluorescent transgenic roots were harvested, then frozen in liquid nitrogen and stored at −80 °C. For this experiment seven biological replicates, each one containing roots from four different composite plants, were included.

2.5. Gene Expression Analysis

To analyze the expression of the *AGO5, NSP2, NIN, FLOT2*, and *ENOD40* genes, total RNA was extracted from 0.5 g of rhizobia-inoculated or mock-inoculated roots using ZR Plant RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. Genomic DNA (gDNA) was removed from purified RNA by using DNaseI RNase-free (Thermo Fisher Scientific) according to manufacturer's instructions. 1 µg of gDNA-free total RNA was used to synthesize complementary DNA (cDNA) using Thermo Scientific RevertAid Reverse Transcriptase (Thermo Fisher Scientific) according to manufacturer's instructions. The cDNA samples were used to analyze the expression of the above-mentioned genes by quantitative real-time PCR (qRT-PCR) in a Step-One qPCR thermocycler (Applied Biosystems, Foster, CA, USA). The housekeeping genes *PvActin* (for common bean; Phvul.008G011000.1) or *Cons6* and *Cons16* (for soybean) [40] were used to normalize gene expression levels. The expression level of different genes was calculated according to the equation $E = P_{eff}^{(-\Delta Ct)}$. P_{eff} is the primer set efficiency calculated using LinRegPCR program [41] and Δ cycle threshold (Ct) was calculated by subtracting the Ct value of the housekeeping gene from the Ct values of a given gene. The nucleotide sequences of the qRT-PCR primers used in this study are provided in Table S1. For this experiment, three biological replicates were analyzed.

2.6. AGO5 Protein Accumulation in Response to Rhizobia

To detect the accumulation of AGO5 protein in response to rhizobia, 0.3 g of fresh rhizobia-inoculated roots (see Treatment section for details) was ground in 0.5 mL of extraction buffer (50 mM $Na_4P_2O_7$, 1 mM Na_2MoO_4, 25 mM NaCl, 10 mM EDTA-Na, 0.5% PVP, 250 mM Sucrose, 50 mM HEPES, 5% glycerol, pH 7.5) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Total protein extract was centrifuged at 12,000 g for 5 min at 4 °C. Proteins were separated by 10% SDS-PAGE, and then transferred to Amersham Protan 0.2 µm nitrocellulose blotting membranes (GE Healthcare Life Sciences, Pittsburgh, PA, USA) by electroblotting. Detection of AGO5 was performed by probing membrane with anti-AGO5 antibody (Agrisera, Vännäs, Sweden; 1:1500

dilution) followed by the use of anti-IgG rabbit-HRP polyclonal antibodies (1:5000; Sigma-Aldrich). Equal loading of proteins between samples was confirmed by Coomassie blue staining. The intensity of the bands detected by western blot was quantified by densitometry using the ImageJ software [42], and the inoculated/un-inoculated ratios were obtained.

2.7. Root Hair Deformation Analysis

Common bean or soybean composite plants, expressing the control vector or *AGO5*-RNAi construct and growing in 25 cm × 25 cm Petri dishes containing Fahräeus medium, were inoculated with 1 mL of saturated (O.D = 1) rhizobia suspension (*R. tropici* for common bean or *B. diazoefficiens* for soybean). Forty-eight hours after inoculation, tdTomato-positive transgenic roots were collected and stained with methylene blue to maximize contrast, and then observed with a bright field microscope. A total of 15 independent biological replicates were generated, each one including ten plants.

2.8. Nodulation Assay

Common bean or soybean composite plants expressing the control vector or the *AGO5*-RNAi construct were transferred into pots with wet agrolite. After five-days of transplanting, common bean or soybean roots were inoculated with 3 mL of *R. tropici* or *B. diazoefficiens*, respectively. Inoculated composite plants were kept in a green house at 25–27 °C. Four weeks after inoculation, composite plants were removed from pots and those nodules developed on tdTomato-positive transgenic roots were counted. Five independent biological replicates, each one including ten plants, were generated.

2.9. Histology of Nodules by Light Microscopy

Images of ten whole transgenic nodules were captured using a SZX10 stereomicroscope (Olympus) equipped with an Olympus UC50 camera (Olympus). Nodule samples were sequentially dehydrated for two hours in 30%, 50%, 90% ethanol, followed by 3 treatments with 100% ethanol, with absolute ethanol-xylene (75–25%, 50–50%, 25–75%, by two hours each) and finally with 100% xylene. Upon dehydration, nodules were incubated for 24 h in xylene-paraplast (50%/50%) before embedded in LR-White resin. Semi-thin sections (25 µm) were prepared using a hand-microtome and stained with safranine in 80% ethanol. Safranine-stained semi-thin sections were examined with a NIKON ECLIPSE E200 bright-field microscope and pictures were obtained with NIS ELEMENTS BR 3.2 software (Nikon Instruments Inc., Melville, NY, USA). Representative photographs of control vector or *AGO5*-RNAi nodules are shown.

2.10. Sequence Collection and Phylogenetic Analysis

We performed a BLAST search to identify AGO family members in *G. max*, *M. truncatula*, and *P. vulgaris* based on the most recent release of their gene annotations (*Wm82.a2.v1*, *P. vulgaris v2.1* and *Mt4.0v2*). BLAST analyses were conducted using *AGO5* from soybean (*GmAGO5*) (Gm.11G190900) as a query. Potential family members were searched and validated using two BLAST resources: Phytozome and HMMER. Applying a stringent cutoff (e-value < e^{-100}), we identified 10, 23, 20 and 14 *AGO* genes in Arabidopsis, soybean, *M. truncatula* and common bean genomes, respectively. The AGO proteins were validated based on the presence of the conserved Piwi and PAZ domains using Interpro bioinformatics resources [43].

In addition, to better understand the evolution of this gene family, we also included the *A. thaliana* AGO family members in our phylogenetic analysis. The phylogenetic relationships between legume and Arabidopsis *AGO* genes were established using the multiple alignment software "Molecular Evolutionary Genetic Analysis" (MEGA) [44]. Bootstrap analyses of 100 resampling replicates were made to test for the statistical significance of nodes.

2.11. Statistical Analyses

All the statistical analyses were conducted using R software 3.0.1 (The R project for Statistical computing). The specific statistical tests performed are indicated in the legend of the corresponding figures.

3. Results

3.1. AGO5 Is Preferentially Expressed in Roots and Nodules of Common Bean Plants

Transcriptomic analyses provide an overview of the plant transcriptional responses to any developmental and environmental stimuli. Moreover, these types of analyses also represent an excellent source to identify new potential regulators of a given biological process. In order to identify new regulators of the symbiosis between legumes and nitrogen-fixing rhizobia, we conducted a data-mining analysis on transcriptional data from *P. vulgaris* interacting with rhizobia.

Our data-mining analysis on the *P. vulgaris* Gene Expression Atlas [33], allowed us to identify several candidate genes, among them Phvul.011G088200.1, predicted to encode AGO5 protein. Based on available transcriptional data in common bean, this gene shows high expression in roots (including root tips) and pods, followed by nodules, leaves, and flowers (Figure S1). To validate these transcriptomic data, we evaluated the expression of this gene by qRT-PCR (Figure 1a). These quantitation analyses revealed that the Phvul.011G088200.1 gene is preferentially expressed in nodules and roots from common bean plants.

AGO5 protein from *A. thaliana* (AT2G27880; *At*AGO5) has seven domains: Argonaute *N*-terminal, Argonaute Linker1, PAZ, Argonaute Linker2, Argonaute Mid, Ribonuclease H-like, and PIWI (Figure S2). To confirm the evolutionary relationships between Phvul.011G088200.1 and *At*AGO5 proteins, we conducted a protein domain and a phylogenetic analysis (Figure S2). Comparison of *At*AGO5 and Phvul.011G088200.1 amino acid sequences showed a 60% identity between them. Furthermore, our protein domain analysis revealed that the AGO5 protein encoded in Phvul.011G088200.1 carries all the characteristic domains of AtAGO5, except the Mid domain (Figure S2). Additionally, our phylogenetic analysis showed that the protein encoded in the gene Phvul.011G088200.1 can be grouped in the *At*AGO5 clade. Altogether, these data indicate that the Phvul.011G088200.1 gene encodes for a *P. vulgaris* AGO5 (*Pv*AGO5) protein, preferentially expressed in roots and nodules of common bean plants.

3.2. PvAGO5 Expression Is Induced in Response to Rhizobia

Because *AGO5* is preferentially expressed in roots and nodules, we hypothesized that the expression of *AGO5* might be activated at early stages of the symbiosis between legumes and rhizobia. To test this hypothesis, we evaluated the expression of *AGO5* in common bean roots inoculated with rhizobia at various time points (1, 3, 6, 12, 24 and 48 h) (Figure 1b and Figure S3). Our expression analysis revealed that upon bacteria inoculation, *AGO5* transcript accumulates more than 2-fold during the first three hours, followed by a decrease between 6 and 48 h after bacteria inoculation (Figure 1b and Figure S3). To look at the correlation between these transcriptomic and AGO5 protein levels, we performed an immunoblotting analysis using AGO5 specific antibodies. This analysis revealed that AGO5 protein accumulation (2-fold) is detected after one hour of rhizobia inoculation, consistent with its relative transcript accumulation timing (Figure 1c). After six hours post-inoculation, a second wave of AGO5 protein accumulation was detected, this higher relative accumulation levels seems to be maintained up to 24 h after rhizobia inoculation (Figure 1d). This transcript and protein accumulation patterns indicate that AGO5 is required for both early and late stages of common bean and rhizobia symbiosis.

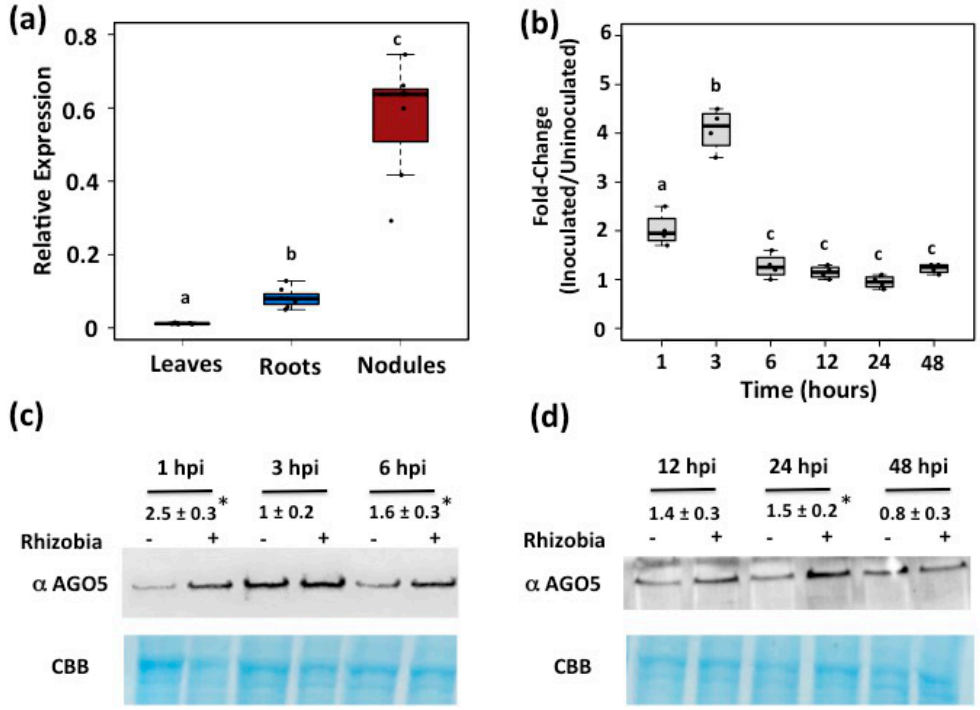

Figure 1. *AGO5* from *P. vulgaris* (*PvAGO5*) is preferentially expressed in root and nodules. (**a**) Expression pattern of *PvAGO5* in leaves, roots and nodules from three weeks old common bean plants; (**b**) Expression profile of *PvAGO5* in rhizobia-inoculated roots from two days old common bean plants. Box plots represent first and third quartile (horizontal box sides), minimum and maximum (outside whiskers). Data shown was obtained from four independent biological replicates. One-way ANOVA followed by a Tukey Honest Significant difference (HSD) test was performed (p-value < 0.01). Statistical classes sharing a letter are not significantly different. (**c**,**d**) AGO5 protein expression in rhizobia-inoculated roots from two days old common bean plants. Immunoblot shown is a representative figure from three biological replicates. The intensity of the bands was quantified densitometrically, and the inoculated/un-inoculated expression ratios were obtained for each time point. Values are mean and standard error of three biological replicates. Asterisks indicate a significant difference according to Student's t-test (p-value < 0.01). hpi = hours post-infection.

3.3. AGO5 Is Required for Rhizobia-Induced Root Hair Deformation and the Activation of Symbiosis-Specific Genes

Upon NFs perception by NFR5 and NFR1, different molecular and physiological responses are triggered [4], including the activation of *Early Nodulin* (*ENOD*) genes and the deformation of the root hair cell [5,45]. Because *AGO5* is expressed during the first three hours after inoculation with rhizobia, we thus hypothesized that *AGO5* might be involved in the control of some of the early steps of the symbiosis between common bean and rhizobia. To test this hypothesis, we first designed an RNAi construct targeting *PvAGO5* and utilized *A. rhizogenes*-mediated transformation to knockdown *PvAGO5*. The expression of *PvAGO5* in common bean transgenic roots expressing the RNAi construct was reduced on an average by 60% compared to roots transformed with a control vector (Figure 2a). To test whether the reduction in the expression of *PvAGO5* affects the rhizobia-induced root hair deformation, common bean transgenic roots expressing either *PvAGO5*-RNAi or control vector were inoculated with *R. tropici* CIAT899. Forty-eight hours after inoculation, 95% (n = 60) of the control vector-transformed roots and 20% (n = 60) of the *PvAGO5-RNAi*-transformed roots showed the characteristic rhizobia-induced root hair deformation (Figure 2b–d).

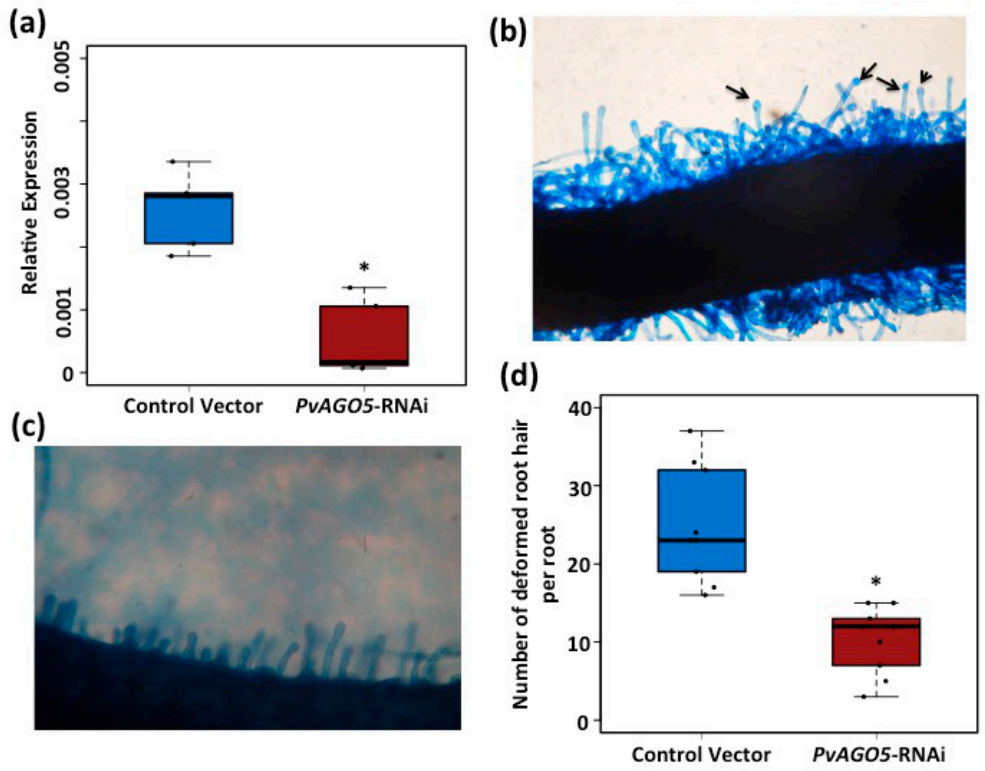

Figure 2. Down-regulation of *PvAGO5* reduces the rhizobia-induced root hair deformation in common bean. (**a**) *PvAGO5* expression levels in transgenic roots expressing a control vector or the *PvAGO5*-RNAi construct. Data shown was obtained from five independent biological replicates, each one containing roots from four different composite plants; (**b**) Rhizobia-induced root hair deformation in common bean transgenic roots expressing a control vector or (**c**) the *PvAGO5*-RNAi, black arrows indicate characteristic rhizobia-induced root hair deformation; (**d**) Number of rhizobia-induced root hairs observed in control transgenic roots and *PvAGO5*-silenced roots. One-way ANOVA followed by a Tukey HSD test was performed. Asterisk indicates a significant difference (p-value < 0.01).

The fact that *PvAGO5* is up-regulated during the first three hours following rhizobia inoculation and that the down-regulation of *PvAGO5* reduces the rate of rhizobia-induced root hair deformation, suggest that *PvAGO5* participates in promoting some the early molecular events leading to nodule development, including the transcriptional activation of *ENOD* genes. To further investigate the molecular role played by *PvAGO5*, we evaluated the expression of the symbiosis-related genes: Nodulation-signalling pathway2 (*PvNSP2*), Nodule inception protein (*PvNIN*), Flotillin2 (*PvFLOT2*), and Early nodulin40 (*PvENOD40*) in common bean transgenic roots expressing either the *PvAGO5*-RNAi construct or the control vector and inoculated for one hour with *R. tropici* (Figure 3). Our expression analysis revealed that the expression of these symbiotic genes in response to rhizobia was reduced by an average of 50% in *PvAGO5*-RNAi roots compared to the roots transformed with the control vector (Figure 3). Together, these results indicate that *PvAGO5* is involved in controlling the expression of some of the major regulatory genes, whose products participate during the early events of common bean-rhizobia symbiosis.

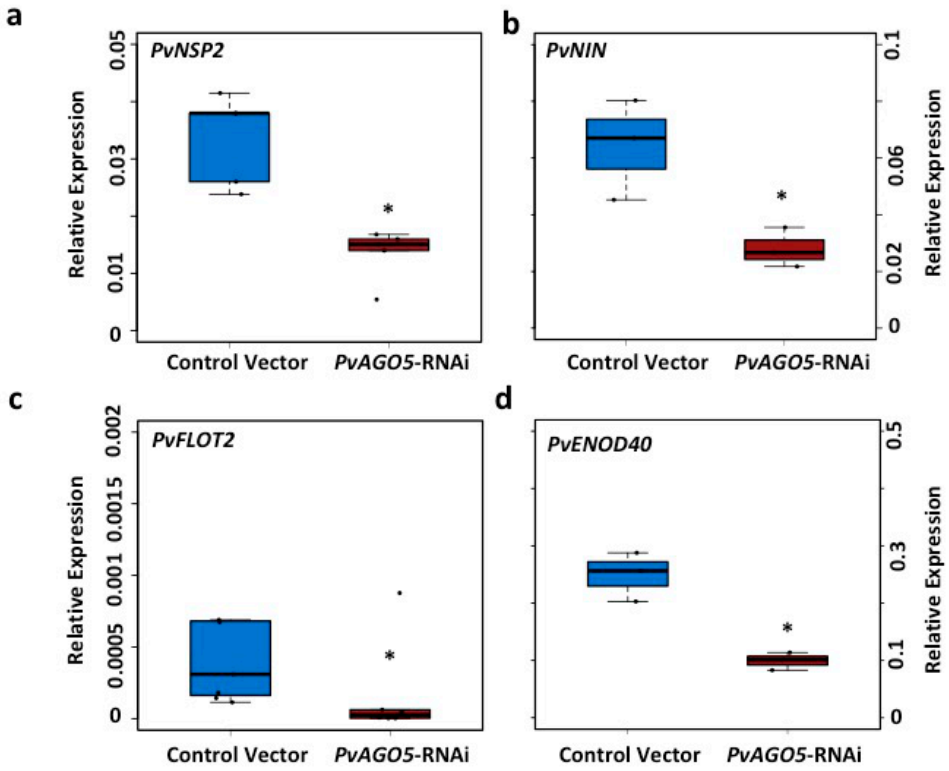

Figure 3. Down-regulation of *PvAGO5* affects the expression of symbiotic genes. Rhizobia-triggered expression of (**a**) Nodulation-signalling pathway2 (*PvNSP2*); (**b**) Nodule inception protein (*PvNIN*); (**c**) Flotillin2 (*PvFLOT2*) and (**d**) Early nodulin40 (*PvENOD40*) in control- and *PvAGO5*-silenced common bean transgenic roots. Data shown was obtained from six independent biological replicates, each one containing four transgenic roots from the same number of composite plants. One-way ANOVA followed by a Tukey HSD test was performed. Asterisk indicates a significant difference (p-value < 0.01).

3.4. Down-Regulation of PvAGO5 Affects Nodule Development in Common Bean

The relative high expression of *PvAGO5* detected in common bean mature nodules (Figure 1a) suggests that PvAGO5 might also play a role during nodule development. To test whether the down-regulation of *PvAGO5* affects the development of common bean nodules, we conducted a nodulation assay on *PvAGO5*-RNAi transgenic roots (Figure 4). Down-regulation of *PvAGO5* resulted in 60% reduction in the nodule number in silenced roots (Figure 4a). Interestingly, those nodules that reach maturity in the *PvAGO5*-silenced roots were irregular, smaller and white, in contrast to the round, large and pink nodules formed in the transgenic roots expressing the control vectors (Figure 4b and Figure S5).

To examine the structural characteristics of the nodules formed in the transgenic roots expressing *PvAGO5*-RNAi, we observed semi-thin sections of *PvAGO5*-RNAi and control vector nodules stained with safranin under a light microscope (Figure 4c,d). Control vector nodules showed the characteristic outer and inner cortexes, the nodule vascular bundles, and the central tissue that contains infected and uninfected cells (Figure 4c). In contrast, *PvAGO5*-RNAi nodules showed a clear different structure with fewer infected cells (Figure 4d). Altogether, these results indicate that the down-regulation of *PvAGO5* significantly affects common bean nodule development and rhizobia colonization.

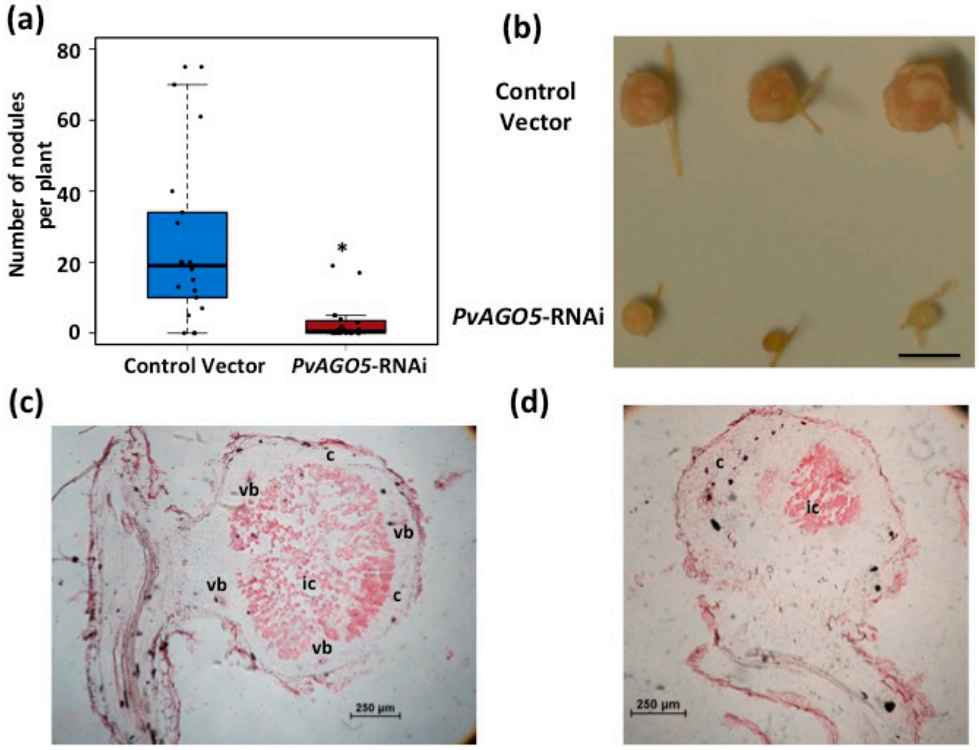

Figure 4. *PvAGO5*-silenced roots develop less, small and white nodules in common bean. (**a**) Nodulation assay on control- and *PvAGO5*-silenced common bean transgenic roots. Data shown was obtained from 30 independent biological replicates. One-way ANOVA followed by a Tukey HSD test was performed. Asterisk indicates a significant difference (p-value < 0.01); (**b**) Nodules observed in control- and *PvAGO5*-silenced common bean transgenic roots. (**c**,**d**) Safranine-stained sections of *R. tropici*-inoculated nodules showing the morphology and organization of representative samples collected from transgenic control (**c**) and *PvAGO5*-RNAi (**d**) roots. c: Cortex; ic: infected cells; vb: vascular bundle.

3.5. AGO5 Is Also Required in Soybean to Establish Symbiosis with B. japonicum

Based on the evident effect of AGO5 on nodule development, we investigated whether this effect could be extrapolated to other legumes. For this, we examined the Soybean Knowledge Base [34,35], and found that Glyma.11g190900.1 gene encodes a putative AGO5 protein. The predicted protein AGO5 soybean protein contains the seven characteristics domains present in *At*AGO5 (Figure S2), and groups in the same clade as *At*AGO5 and *Pv*AGO5 (Figure S2). Similarly to *PvAGO5*, *GmAGO5* transcript was highly accumulated in soybean nodules and roots (Figure 5a and Figure S4), as well as in roots after one hour of *B. diazoefficiens* inoculation, this pattern was similar for *Gm*AGO5 protein accumulation level (Figure 5c,d). Despite these similarities between common and soybean, the protein levels of *Gm*AGO5 were significantly lower three hours after rhizobia inoculation in soybean. Even the second wave of AGO5 protein accumulation detected six hours after rhizobia inoculation in common bean was not observed in soybean (Figures 1d and 5d). These differences in the accumulation of AGO5 proteins in response to rhizobia might be due to intrinsic differences in the way that these two legumes communicate with their symbionts.

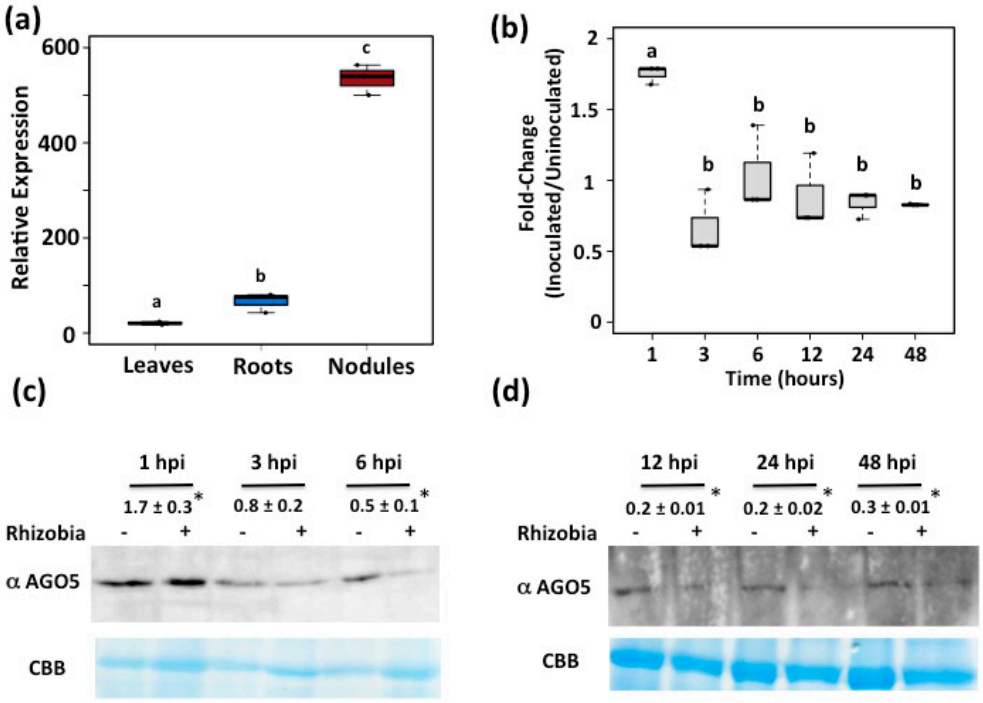

Figure 5. *AGO5* is preferentially expressed in soybean roots and nodules. (**a**) Expression pattern of *GmAGO5* in leaves, roots and nodules from three weeks old soybean plants; (**b**) Expression profile of *GmAGO5* in rhizobia-inoculated root from two days old soybean plants. Box plots represent first and third quartile (horizontal box sides), minimum and maximum (outside whiskers). Data shown was obtained from four independent biological replicates. One-way ANOVA followed by a Tukey HSD test was performed (p-value < 0.01). Statistical classes sharing a letter are not significantly different; (**c**,**d**) AGO5 protein expression in rhizobia-inoculated roots from two days old soybean plants. Immunoblot shown is a representative figure from three biological replicates. The intensity of the bands was quantified densitometrically, and the inoculated/uninoculated expression ratios were obtained for each time point. Values are mean and standard error of three biological replicates. Asterisks indicate a significant difference according to Student's t-test (p-value < 0.01). hpi = hours post-infection.

Because we observed that *GmAGO5* showed a similar expression pattern than *PvAGO5* in response to rhizobia, we also generated an *GmAGO5*-RNAi construct to silence *GmAGO5* in transgenic soybean roots produced by *A. rhizogenes*-mediated transformation. The expression of *GmAGO5* in soybean transgenic roots expressing the RNAi construct was reduced on an average by approximately 50% compared to the transcript accumulation obtained for roots transformed with a control vector (Figure 6a). To test whether the reduction in the expression of *GmAGO5* affects the typical rhizobia-induced root hair deformation, soybean transgenic roots expressing either *GmAGO5*-RNAi or control vector were inoculated with *B. diazoefficiens* USDA110. Although *GmAGO5*-RNAi transgenic roots showed characteristic rhizobia-induced root hairs, we observed that these *GmAGO5*-silenced roots predominantly exhibit "spatula-like" root hairs (Figure 6b). This root hair phenotype was observed only in *GmAGO5*-RNAi transgenic roots inoculated with *B. diazoefficiens*, indicating that this phenotype is dependent on symbiotic signaling.

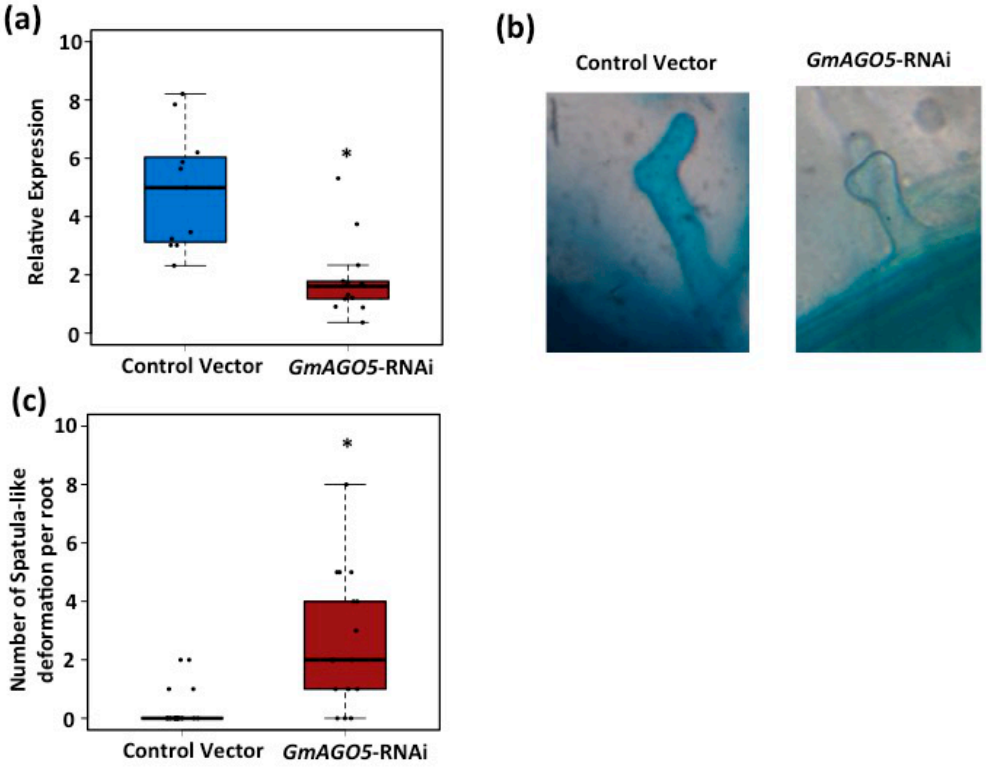

Figure 6. *GmAGO5*-silenced soybean roots develop rhizobia-induced spatula-like root hair deformation. (**a**) *GmAGO5* expression levels in transgenic roots expressing a control vector or the *GmAGO5*-RNAi construct. Data shown was obtained from ten independent biological replicates, each one containing roots from four different composite plants; (**b**) Rhizobia-induced root hair deformation in transgenic soybean roots expressing a control vector or the *GmAGO5*-RNAi construct; (**c**) Number of rhizobia-induced spatula-like deformed root hairs observed in control transgenic roots and *GmAGO5*-silenced roots. One-way ANOVA followed by a Tukey HSD test was performed. Asterisk indicates a significant difference (p-value < 0.01).

To explore if *GmAGO5* also plays a role during nodule development similar to *PvAGO5*, we conducted a nodulation assay on soybean transgenic roots expressing *GmAGO5*-RNAi or the control vector. This assay revealed that *GmAGO5*-silenced roots developed 50% less nodules than control vector roots (Figure 7a). Similar to RNAi-*PvAGO5* nodules, the nodules formed on the *GmAGO5*-silenced transgenic roots were smaller and white, indicating a lack of leghemoglobin (Figure 7a and Figure S5). Light microscopy analysis of transgenic nodule semi-thin sections stained with safranin staining revealed that *GmAGO5*-RNAi nodules contain less infected cells than control vector-transformed nodules. These results also indicate that, similar to *PvAGO5*, silencing of *GmAGO5* results in the reduction in root hair deformation, along with reduced nodule formation efficiency and nodule morphology defects. Altogether, our data indicate that *AGO5* is playing a central role in the control of early events (i.e., expression of *NSP2*, *NIN* and *FLOT2* genes expression and rhizobia-induced root hair deformation) allowing rhizobia infection and proper development of common bean and soybean nodules.

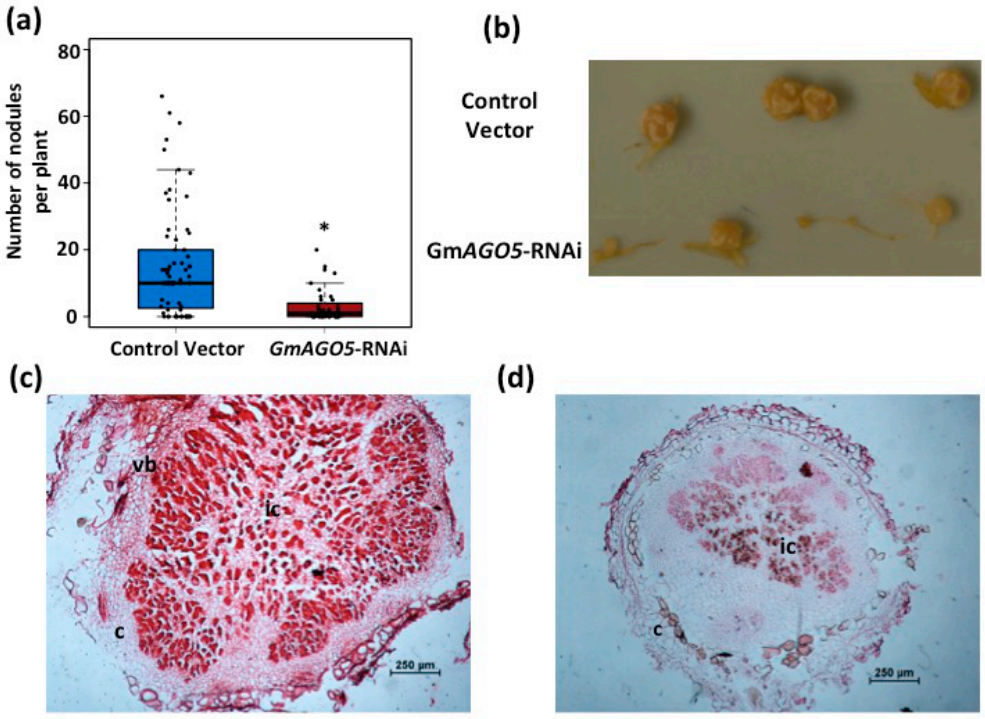

Figure 7. Down-regulation of AGO5 affects nodule development in soybean. (**a**) Nodulation assay on control- and *GmAGO5*-silenced common bean transgenic roots. Data shown was obtained from 30 independent biological replicates. One-way ANOVA followed by a Tukey HSD test was performed. Asterisk indicates a significant difference (p-value < 0.01); (**b**) Nodules observed in control- and *PvAGO5*-silenced common bean transgenic roots. (**c**,**d**) Safranine-stained sections of *B. japonicum*-inoculated nodules showing the morphology and organization of representative samples collected from transgenic control (**c**) and *GmAGO5*-RNAi (**d**) roots. c: Cortex; ic: infected cells; vb: vascular bundle.

4. Discussion

The symbiosis between legumes and rhizobia has been extensively studied. However, transcriptomic, proteomic and even phosphoproteomic analyses have uncovered the existence of potential new regulators of this important symbiosis [30–32]. Nevertheless, few of them have been functionally characterized and assigned a role in the establishment of this process [46–49]. In the present study, we provide evidence supporting the participation of AGO5 in the regulation of both early and late symbiotic processes in common bean and soybean, two major legume crops. We demonstrated that the expression of *AGO5* is induced during the first three hours of rhizobia inoculation. Further experimentation on *PvAGO5*-silenced common bean roots revealed that the rhizobia-induced root hairs deformation and the expression of *PvNSP2*, *PvNIN*, *PvFLOT2* and *PvENOD40* symbiosis-related genes were notoriously affected. Accordingly, we showed that *PvAGO5*-silenced common bean transgenic roots developed 50% less nodules and their nodules were smaller with few infected cells compared to the control transgenic roots. The effect of the down-regulation of *AGO5* in the symbiosis with rhizobia was also observed in soybean *GmAGO5*-silenced roots. These results led us to propose that AGO5 is an essential component in the establishment of the symbiosis with rhizobia in determinate legumes.

Like other AGO proteins, AGO5 binds to different types of non-coding small RNAs, particularly those initiating with cytosine, to form RISC, the complex mediating the transcriptional and posttranscriptional gene silencing [50]. The *AGO5* gene is present in most land-plants and its expression pattern is likely plant-species specific [51]. Additionally, AGO5 has been involved in the regulation of the systemic resistance of *A. thaliana* against Potato Virus X [52]. There is also evidence indicating

that the *AGO5* expression is activated by different abiotic stresses, including drought and salinity in apples [51]. Recently, it was demonstrated that the soybean seed pigmentation is controlled by AGO5-associated small interference RNAs targeting the chalcone synthase transcripts [53]. Here we reported an additional AGO5 function, which might be legume-specific. However, we do not exclude the possibility that AGO5 may also play a role in the interaction of non-legume plants with soil beneficial microbes.

Early molecular responses activated upon NFs perception are critical for a successful symbiosis between legumes and rhizobia [4,5]. Some of these early responses include: protein phosphorylation [3], rapid oscillations in the nuclear and perinuclear calcium concentration (calcium spiking) [10], the synthesis and accumulation of mevalonate [17] and the activation of different *NOD* genes [3]. These early molecular responses, in turn, are finely regulated by a set of genes that altogether conform the so-called Common Symbiosis Pathway (CSP) [3,9]. One of the characteristics of the CSP participating genes is their preferential expression in roots and their early activation, few hours after NFs perception. These early molecular responses positively control root hair deformation or curling, which is required for rhizobia colonization. In this study, we showed that *PvAGO5*-silenced common bean transgenic roots showed a significant reduction in the rhizobia-induced root hair deformation. However, the deformed roots hairs were similar to those observed in control transgenic roots (Figure 1b). In contrast, *GmAGO5*-silenced soybean roots predominantly exhibited "spatula-like" root hairs (Figure 6). This spatula-like phenotype has also been observed in *M. truncatula ern1/ern2* and *dmi1* mutant plants [36,54]. This defect in the root hair deformation has been associated to an inhibition of the polar elongation of the root hair cell, which affects the formation of the infection chamber and the subsequent rhizobia colonization and nodule formation [36,54,55]. The fact that the *AGO5* expression is activated during the first three hours of interaction with rhizobia and that *AGO5*-silenced transgenic roots show defects in the rhizobia-induced root hair deformation, suggest that *AGO5* has a critical role in the rhizobia colonization by controlling the polar growth of root hairs and the formation of the infection chamber.

PvAGO5-silenced common bean transgenic roots showed 50% less accumulation of *PvNSP2*, *PvNIN*, *PvFLOT2* and *PvENOD40* symbiotic transcripts, which are required for the infection thread formation and rhizobia colonization [56–59]. NSP2 along with NSP1 forms a DNA binding complex regulating the expression of the *NIN* and *ERN1* symbiotic genes which encode transcription factors required for rhizobia infection and colonization [56]. It has been reported that *nsp2 M. truncatula* mutant plants show a reduction in rhizobia-induced root hair deformation and a complete absence of rhizobia infection [60]. In contrast, *M. truncatula nin* mutants show an excessive root hair deformation without rhizobia infection nor nodule formation [56]. Other genes required for rhizobia infection and colonization are *FLOT2* and *FLOT4* [59]. Down-regulation of these two flotillin genes seriously affects the infection thread elongation and nodule formation in *M. truncatula* transgenic roots [59]. It has also been demonstrated that the symbiotic gene *ENOD40*, which is expressed in pericycle-, nodule primordium- and nodule cells, is required for optimal nodule and bacteroid development [57]. Phenotypes similar to those reported in the *nsp2* mutant plants and *FLOTILLIN*-silenced roots were observed in the present study (Figures 3, 4 and 7). Considering that the down-regulation of *AGO5* significantly reduced the expression of *NSP2*, *NIN*, *FLOT2* and *ENOD40*, that the rhizobia-induced root hair deformation was significantly reduced and that the nodules formed in the *AGO5*-silenced roots were smaller and showed few infected cells, these data support our hypothesis that AGO5 is critical for rhizobia colonization. Additionally, because *AGO5*-silenced roots did not show reduction in the expression of the symbiosis-related gene *CYCLOPS* (Figure S6), but genes acting downstream of this transcription factor do (e.g., *NSP2* and *NIN*), with this data it is tempting to speculate that AGO5, along with its associated small RNAs, might act upstream of the NSP2/NSP1 complex. However, further experimentation is needed.

It has been demonstrated that both phased small interfering RNAs (phasiRNAs) and microRNAs, particularly those that with a cytosine at the 5'-end, interact with *AGO5* [50,61]. Additionally,

it has also been reported that miR167 and miR172c are the most abundant microRNAs when AGO5-associated small RNAs were determined by Co-Immuno Precipitation (co-IP) assays in *A. thaliana* [50]. Interestingly, there is evidence indicating that the nodes miR172c-*APETALA2-1* and miR167-*GmARF8* control early events (e.g., rhizobia-induced root hair deformation and the activation of symbiosis-related genes) of this symbiosis and nodule development in common bean and soybean, respectively [62,63]. Hence, it is possible that the defects in the establishment of the symbiosis between common bean/soybean and rhizobia might be due to a misregulation in the activity of AGO5-dependent microRNAs that control symbiosis-related genes.

5. Conclusions

The data presented in this study sheds light on the role of AGO5 in the establishment of the symbiosis between legumes and rhizobia as well as the correct development of functional nodules. However, it is still not clear the role that *AGO5* is playing in this process. One possibility is that some AGO5-associated small RNAs target particular symbiotic genes. Ongoing work in our laboratory is oriented to identify the small RNAs that are associated to AGO5 in common bean and soybean under both symbiotic and non-symbiotic conditions.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4425/8/12/352/s1. Figure S1 Expression profile of *AGO5* from common bean and soybean; Figure S2 Domain and phylogenetic analysis of AGO5 from common bean and soybean; Figure S3 Expression levels of *PvAGO5* in responses to mock or rhizobia inoculation; Figure S4 Expression levels of *GmAGO5* in responses to mock or rhizobia inoculation; Figure S5 *AGO5*-RNAi nodules are small and white; Figure S6 Expression level of *PvCYCLOPs* in *AGO5*-RNAi roots; Table S1 Primers used for qRT-PCR analysis.

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Author Contributions: O.V.-L., M.L., J.L.R. and A.A.C. proposed and designed the study M.d.R.R.-S. performed the gene and protein expression analysis, generated composite plants, nodulation and root hairs deformation assay, nodule histology and analyzed the data. M.d.S.S.-C. generated the RNAi constructs, performed part of the gene and protein expression and rhizobia-induced root hair deformation. Z.Q. performed the bioinformatics analyses, generated the *AGO5* phylogenetic analysis. M.E.D.-P. performed part of the nodulation assay. M.d.R.R.-S., M.L. and O.V.-L. wrote the manuscript. All authors read and approved the manuscript.

Conflicts of Interest: The authors declare no conflict of interest

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Argonaute Proteins: Why Are They So Important for the Legume–Rhizobia Symbiosis?

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Unlike most other land plants, legumes can fulfill their nitrogen needs through the establishment of symbioses with nitrogen-fixing soil bacteria (rhizobia). Through this symbiosis, fixed nitrogen is incorporated into the food chain. Because of this ecological relevance, the genetic mechanisms underlying the establishment of the legume–rhizobia symbiosis (LRS) have been extensively studied over the past decades. During this time, different types of regulators of this symbiosis have been discovered and characterized. A growing number of studies have demonstrated the participation of different types of small RNAs, including microRNAs, in the different stages of this symbiosis. The involvement of small RNAs also indicates that Argonaute (AGO) proteins participate in the regulation of the LRS. However, despite this obvious role, the relevance of AGO proteins in the LRS has been overlooked and understudied. Here, we discuss and hypothesize the likely participation of AGO proteins in the regulation of the different steps that enable the establishment of the LRS. We also briefly review and discuss whether rhizobial symbiosis induces DNA damages in the legume host. Understanding the different levels of LRS regulation could lead to the development of improved nitrogen fixation efficiency to enhance sustainable agriculture, thereby reducing dependence on inorganic fertilizers.

Keywords: argonaute proteins, legumes, symbiosis, microRNAs, small RNAs

INTRODUCTION

The symbiosis between legumes and rhizobia is of considerable ecological importance because through it, fixed nitrogen (e.g., ammonium) is incorporated into the food chain (Castro-Guerrero et al., 2016). In this context, it has been estimated that the legume–rhizobia symbiosis fixes 60 million metric tons of nitrogen worldwide (Smil, 1999). As symbiotic nitrogen fixation also plays essential roles in soil function, nutrient and water cycling, and food security, its exploitation and improvement in crop plants can promote lower input sustainable agriculture (Ferguson et al., 2019a).

To establish this symbiosis, a molecular dialogue between legumes and rhizobia is required (Venkateshwaran et al., 2013). This dialogue implies the interchange of diffusible signals, which includes legume-derived flavonoids and rhizobia-secreted lipochito-oligosaccharides (LCOs) with specific chemical decorations, named Nodulation Factors (NFs) (Dénarié et al., 1996). Upon NFs

perception by the legume host, a series of molecular events is activated, enabling rhizobial infection and nodule formation (Venkateshwaran et al., 2013).

Legume–rhizobia symbiosis (LRS) is regulated at the transcriptional, posttranscriptional, and posttranslational level (Venkateshwaran et al., 2013). For instance, it has been demonstrated that the transcription factor (TF) Nodule Inception (NIN) controls rhizobial root infection, colonization, and nodule formation (Liu CW et al., 2019; Liu J et al., 2019). NIN also activates the expression of the *CLE ROOT SIGNALING1* (*CLE-RS1*) and *CLE-RS2* peptides in *Lotus japonicus* (Soyano et al., 2014). These two CLE peptides participate in the Autoregulation of Nodulation (AON) process, which limits the number of nodules (Ferguson et al., 2019b).

MicroRNAs (miRNAs), which are small regulatory RNA molecules, play a substantial role in the posttranscriptional regulation of LRS (Moran et al., 2017). For example, it has been demonstrated that miRNAs miR166 and miR169 regulate nodule development (Combier et al., 2006; Boualem et al., 2008) in *Medicago truncatula*. However, miRNAs not only regulate nodule development, but they also participate earlier in the rhizobial infection process (Bazin et al., 2012). The involvement of miRNAs, and likely other small RNAs (sRNAs), in the LRS strongly implicates the participation of Argonaute (AGO) proteins, which together form so-called RNA-induced silencing complexes (RISCs). We recently reported that AGO5 participates in the rhizobial infection process in both *Phaseolus vulgaris* (common bean) and *Glycine max* (soybean) (Reyero-Saavedra et al., 2017). Despite this evidence, the involvement of AGO proteins in LRS has been largely overlooked.

Here, we briefly recapitulate the genetic control of LRS by TFs and miRNAs. Likewise, based on the role of different small RNAs (sRNAs) and some AGO proteins in the regulation of both plant development and plant–pathogen interactions, we hypothesize the stages of this symbiosis where AGO proteins might play a role. Finally, we also discuss whether rhizobial symbiosis causes DNA damage in the legume host. By improving our understanding of the different levels of LRS regulation, we may be able to enhance symbiotic nitrogen fixation efficiency in crop legumes.

GENETIC REGULATION OF LEGUME–RHIZOBIA SYMBIOSIS

NFs are detected by two LysM-type receptor kinases, named NFs Perception (NFP) and LysM-domain Receptor-Like Kinase3 (LYK3), in *M. truncatula* and NFs Receptor5 (NFR5) and NFR1 in *L. japonicus* (Limpens et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006). Both NFP/NFR5 and LYK3/NFR1 receptors have a similar structure, which includes an extracellular domain composed of three LysM domains, a transmembrane domain, and an intracellular kinase domain. These two receptors are essential for legume–rhizobial communication, and they may have evolved independently from two different ancestral receptors, which were likely involved in the perception of Mycorrization (Myc)-LCOs (De Mita et al., 2014). Myc-LCOs are signal molecules released by endomycorrhizal fungi and are required for most land plants to engage in symbiosis with these beneficial microbes (Maillet et al., 2011). Interestingly, Myc-LCOs and NFs are structurally very similar, which reinforces the hypothesis that NF receptors evolved from ancestral receptors involved in the perception of Myc-LCOs. The evolution of the NF's extracellular domain arguably provided high specificity to the rhizobial symbiosis; it has been demonstrated that the evolution of the second LysM domain contributed to ligand binding, whereas the first LysM domain contributed to ligand specificity (De Mita et al., 2014).

Upon perception of NFs *via* the receptors NFP/NFR5 and LYK3/NFR1, a series of molecular events, including protein phosphorylation, are triggered (Broghammer et al., 2012). The phosphorylation of proteins is crucial to decipher the NFs signal. For example, one of the phosphorylated proteins playing a role in this symbiosis is 3-hydroxy-3-methylglutaryl coenzyme A reductase1 (HMGR1) (Kevei et al., 2007). HMGR1 participates in mevalonate biosynthesis, and it has been demonstrated that mevalonate is sufficient to trigger calcium oscillations in the nuclear region, also known as calcium spiking (Venkateshwaran et al., 2015). Calcium spiking is a crucial signature to establish rhizobial symbiosis. Membrane ion channel mutants, such as *L. japonicus castor* and *pollux* and the *M. truncatula* mutant that *does not make infections1* (*dmi1*), are unable to activate calcium spiking and therefore fail to nodulate (Imaizumi-Anraku et al., 2005).

Calcium spiking is further decoded by a calcium/calmodulin (Ca^{+2}/CaM)-dependent protein kinase (CCaMK/DMI3) (Lévy et al., 2004). Upon activation, CCaMK/DMI3 immediately phosphorylates the transcriptional activator Interacting Protein of DMI3 (IPD3)/CYCLOPS (Singh et al., 2014). In turn, IPD3/CYCLOPS activates the expression of *NIN*, which subsequently promotes the expression of the *Nuclear Factor Y* (*NF-Y*) complexes *NF-YA* and *NF-YB* (Soyano et al., 2013). The coordinated action of these TFs and the interplay of the TF Nodulation Signaling Pathway2 (NSP2)/NSP1, Ethylene Response Factor Required for Nodulation1 (ERN1), and ERN2 lead to the transcriptional activation of symbiosis-related genes participating in the rhizobial infection process (Genre and Russo, 2016). Some of the genes activated by this transcriptional node are *Early Nodulin11* (*ENOD11*), which is involved in the infection processes (Journet et al., 2001), and the *Flotillins* (*FLOT*) *FLOT2* and *FLOT4*, which are involved in the formation of the infection thread, a tubular structure essential for rhizobial infection of the root cells (Haney and Long, 2010) (**Figure 1**).

In parallel with the activation of the molecular events leading to the infection/colonization of the root by the rhizobia, legumes activate a second genetic program that is required for nodule development (Oldroyd et al., 2011; Plet et al., 2011). It has been demonstrated that a delicate balance between the phytohormones auxin and cytokinin activates this genetic program (Hirch et al., 1989; Van Zeijl et al., 2015; Gamas et al., 2017). The activation of this genetic program begins with the inhibition of polar auxin transport, which leads to the accumulation of cytokinins in root cortical cells (Nadzieja et al., 2018). Cytokinins are then detected in root cortical cells through the receptor Cytokinin Response1 (CRE1)/Lotus Histidine Kinase1 (LHK1) (Plet et al., 2011). Interestingly, upon cytokinin perception, NIN and NSP2/NSP1 are also activated, controlling the expression of genes involved in the development of the nodule (Madsen et al., 2010).

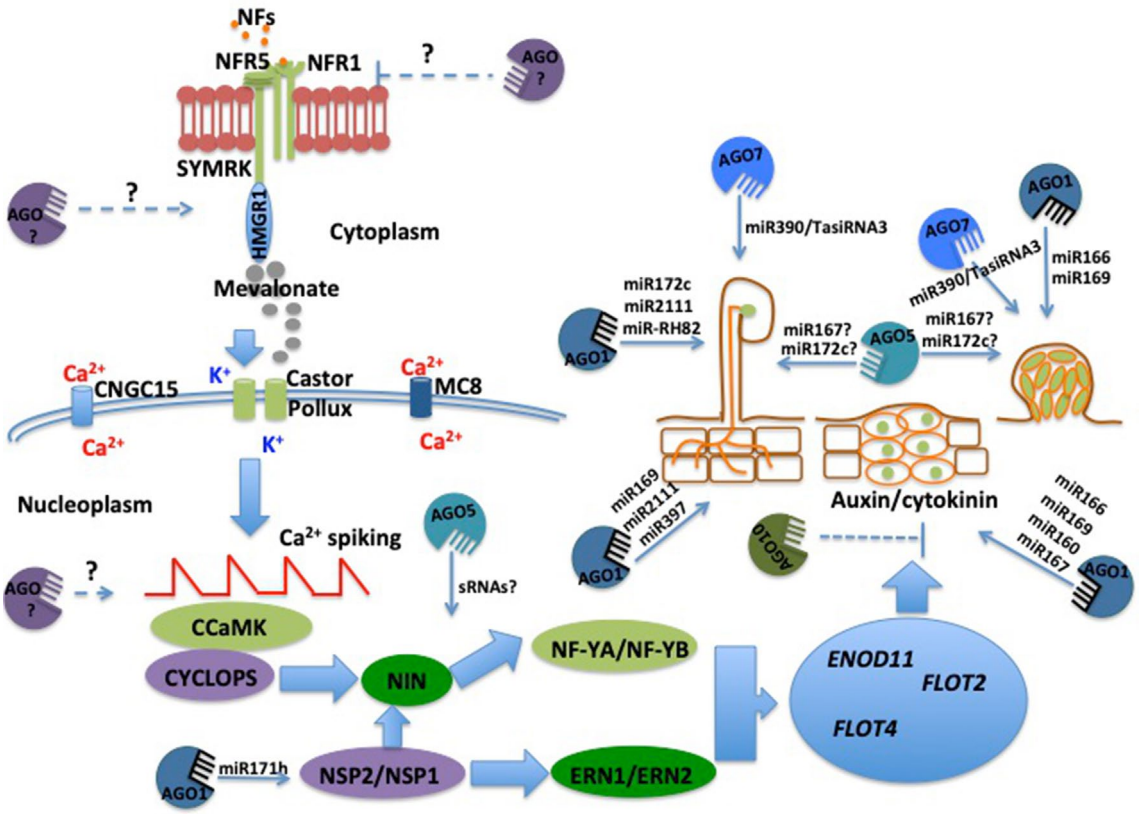

FIGURE 1 | Participation of Argonaute (AGO) proteins in different stages of the legume–rhizobia symbiosis (LRS) According to several reports, different AGO proteins may participate in each stage of the LRS. Although several miRNAs have been identified a few hours upon NF perception, there is no experimental evidence indicating that they regulate very early stages of LRS, such as NFs perception and activation of calcium spiking. However, there is solid evidence supporting the participation of both sRNAs and different AGO proteins in rhizobial infection and the development of both nodule meristems and root nodules. Dashed lines indicate the potential participation of AGO proteins and sRNAs in the LRS.

Although this symbiosis provides fixed nitrogen to the plant, this process demands a significant amount of energy from legumes. Because of this carbon demand, legumes tightly regulate the number of nodules via AON. In *L. japonicus,* AON is systemically regulated by the CLE-RS1 and CLE-RS2 peptides (Soyano et al., 2013; Ferguson et al., 2019b). These two CLE peptides travel from the root to the shoots where they are detected by the receptor Hypernodulation Aberrant Root formation1 (HAR1) (Nishimura et al., 2002). Upon perception of CLE peptides, a signal molecule, likely a shoot-derived cytokinin or the miRNA miR2111, is produced and sent to the roots (Tsikou et al., 2018; Ferguson et al., 2019b). The perception of this shoot-derived molecule in the roots then triggers the inhibition of nodule development.

ROLE OF miRNAS IN THE ESTABLISHMENT OF THE LEGUME–RHIZOBIA SYMBIOSIS

The first miRNAs known to be involved in the LRS were miR169 and miR166, which regulate meristem maintenance, bacterial release, and vascular differentiation in both roots and nodules of *M. truncatula* plants (Combier et al., 2006; Boualem et al., 2008). MiR169 and miR166 regulate these stages of LRS through the modulation of the expression of the TF genes *NF-YA1* (formerly called *HAP2-1* for *HAPLESS2-1*) and *class-III homeodomain-leucin zipper* (*HD-ZIPIII*), respectively (Combier et al., 2006; Boualem et al., 2008; Laloum et al., 2013). Since the publication of these two studies, a large number of symbiosis-responsive miRNAs has been identified in different stages of LRS. For instance, Subramanian et al. (2008) reported many miRNAs that were differentially regulated after 3 h of rhizobial inoculation in soybean (**Table 1**).

Because LRS is initiated in root hairs, Formey et al. (2016) hypothesized that root hair miRNA expression analysis after 6 h of NFs treatment could identify regulators of early events of rhizobial infection. As a result, Formey et al. (2016) identified six symbiosis-responsive miRNAs in the common bean. Interestingly, one of the identified miRNAs was the root hair-specific miR-RH82. This observation suggests that this novel miRNA might play an essential role in the early stages of the LRS (Formey et al., 2016).

Although several studies report differential expression of miRNAs during the first hours of legume–rhizobia interaction, there is limited experimental evidence to indicate that they regulate very early symbiotic events, such as calcium spiking.

TABLE 1 | Differentially regulated plant miRNAs and their corresponding target genes during the early stages of the legume–rhizobia symbiosis.

Reference	hpi	Regulation	miRNA	Target Gene Name
Subramanian et al., 2008	3	Up	miR168	*Argonaute 1*
			miR172	*Apetala 2 like*
			miR159	*Auxin Responsive Factor like*
			miR393	*Transport Inhibitor Response 1*
		Down	miR160	*Auxin Responsive Factor 10,16,17*
			miR164	*NAC domain containing protein 1*
			miR166	*Class III homeodomain leucine zipper*
			miR169	*Nuclear Factor YA-1* (*Hapless 2-1)*
			miR396	*Growth-Regulating Factors*
Formey et al., 2016	6	Up	miR171a	*Nodulation-signaling pathway 2*
			miR398b-3p	*Cu/Zn Superoxide Dismutase 1/Nodulin 19*
		Down	miR171a-3p	*GRAS family transcription factor*
			miR398c	ND
			miR482b-3p	*Nucleotide-Binding Site–Leucine-Rich Repeat*
			miR-RH82	ND

hpi, hours postinoculation; ND, nondetermined target gene.

However, miRNAs have been identified that participate in rhizobial preinfection and infection processes, including miR171c and miR397 in *L. japonicus* (**Figure 1**) (De Luis et al., 2012). Interestingly, miR171c has been shown to target transcripts of the TF gene *NSP2*, which is crucial for the preinfection and infection process (De Luis et al., 2012). To provide evidence supporting the role of these two miRNAs in the rhizobial infection process, De Luis et al. (2012) made use of *L. japonicus snf1* and *snf2* mutants, which produce autoactive versions of the CCaMK and the cytokinin receptor LHK1, respectively. These two mutants can develop nodules in the absence of rhizobia (spontaneous nodules), but they also form infected functional nodules upon rhizobial inoculation (Tirichine et al., 2006a; Tirichine et al., 2006b; Tirichine et al., 2007). By using these mutants, De Luis et al. (2012) demonstrated that miR171c and miR397 significantly accumulate in infected nodules of *snf* mutants but not in spontaneous nodules, suggesting that these miRNAs might play a role in the rhizobial infection process. Another early-acting miRNA is miR172c, which has been demonstrated to target transcripts of the TF gene *APETALA2-1* (*AP2-1*) and plays a role in rhizobia-induced root hair deformation in the common bean (Nova-Franco et al., 2015). In addition, miR172c has also been characterized in soybean, where it acts as a regulator of early nodulins during nodule initiation through the TF Nodule Number Control1 (GmNNC1) (Wang et al., 2014). In the context of the systemic AON mechanism activated upon rhizobial infection, one candidate for the induced shoot-derived inhibitor (SDI) of nodulation could be miR2111, which targets transcripts of the *F-box* gene *Too Much Love*, a crucial regulator of rhizobial infection and nodule number in *L. japonicus* (Tsikou et al., 2018; Ferguson et al., 2019b).

Moving beyond the early stages of infection, several miRNAs participating in nodule development have been reported. To initiate the formation of the nodule meristem and nodule, a delicate balance between auxin and cytokinin is required (Oldroyd et al., 2011; Plet et al., 2011). In soybean plants, miR160 is essential to modulate the levels of these two phytohormones for nodule development (Turner et al., 2013; Nizampatnam et al., 2015). Recently, it has also been demonstrated that the miR390/Trans-Actin Short Interference RNA3 module negatively regulates both rhizobial infection and nodule organogenesis in *M. truncatula* (Hobecker et al., 2017).

ARGONAUTE PROTEINS IN SYMBIOSIS

AGO proteins are present in eukaryotes, and they participate in many biological processes, including interactions with the environment. AGO proteins are characterized by the presence of four domains: a variable N-terminal domain and conserved PAZ (PIWI-ARGONAUTE-ZWILLE), MID (middle), and PIWI domains (Tolia and Joshua-Tor, 2007). The PAZ domain binds sRNAs, whereas the MID domain specifically recognizes the 5' nucleotide of sRNAs. The PIWI domain adopts an RNase H-like fold, enabling most AGO proteins to cleave target messenger RNAs complementary to the bound sRNAs (Song et al., 2004). The number of AGO proteins present in plants is variable and is plant species-dependent (**Figure 2**). For instance, the *Arabidopsis thaliana* genome encodes 10 AGO proteins, whereas the soybean and the common bean genomes encode 22 and 14 AGO proteins, respectively (Liu et al., 2014; Reyero-Saavedra et al., 2017). Despite this diversity of AGO proteins in flowering plants, these proteins can be grouped into three major phylogenetic clades: AGO1/5/10, AGO2/3/7, and AGO4/6/8/9 (**Figure 2**), with AGO1 being the founding member of the *AGO* gene family (Zhang et al., 2015).

Recent evidence indicates that AGO proteins respond to environmental stimuli (Manavella et al., 2019). The direct involvement of plant AGO proteins in biotic interactions is also well known, mainly for plant defense against bacteria and virus

(Raja et al., 2008; Carbonell and Carrington, 2015; Fátyol et al., 2016). However, the role of AGO proteins in the regulation of mutualistic interactions, such as symbiosis, in animals as well as plants is poorly documented. In plants, there is only one report on this topic, which demonstrates the importance of AGO5 in LRS regulation (Reyero-Saavedra et al., 2017). Despite these knowledge gaps, several studies provide evidence that converges on the importance of AGO proteins in LRS. In this section, we aim to compile exhaustive information about legume AGO protein clades and hypothesize roles for some of them in each stage of LRS.

Ago1/5/10 Clade

As many miRNAs have been reported as regulators of different stages of LRS (**Table 2**), AGO1 is clearly involved in this symbiotic process. For example, very recently, it was demonstrated that soybean AGO1 is hijacked by three rhizobial tRNA-derived small RNA fragments to regulate the expression of three plant genes involved in both rhizobial infection and nodule development (Ren et al., 2019). Other members of the AGO1/5/10 clade may also be involved in LRS regulation. The clearest evidence of AGO regulation of nodulation involves *AGO5* (Reyero-Saavedra et al., 2017), which is upregulated 3 h after rhizobial inoculation in common bean and soybean roots. Furthermore, AGO5 is required for rhizobia-induced root hair deformation and nodule development (Reyero-Saavedra et al., 2017). One possible explanation for this comes from *A. thaliana*, in which AGO5 associates with miR167 and miR172c (Mi et al., 2008). In legumes, miR167 and miR172c have been shown to participate in nodule development through the regulation of the *AUXIN RESPONSE FACTOR8* and *AP2-1* genes, respectively (Nova-Franco et al., 2015; Wang et al., 2015). Beyond the well-studied AGO1 protein, AGO5 is

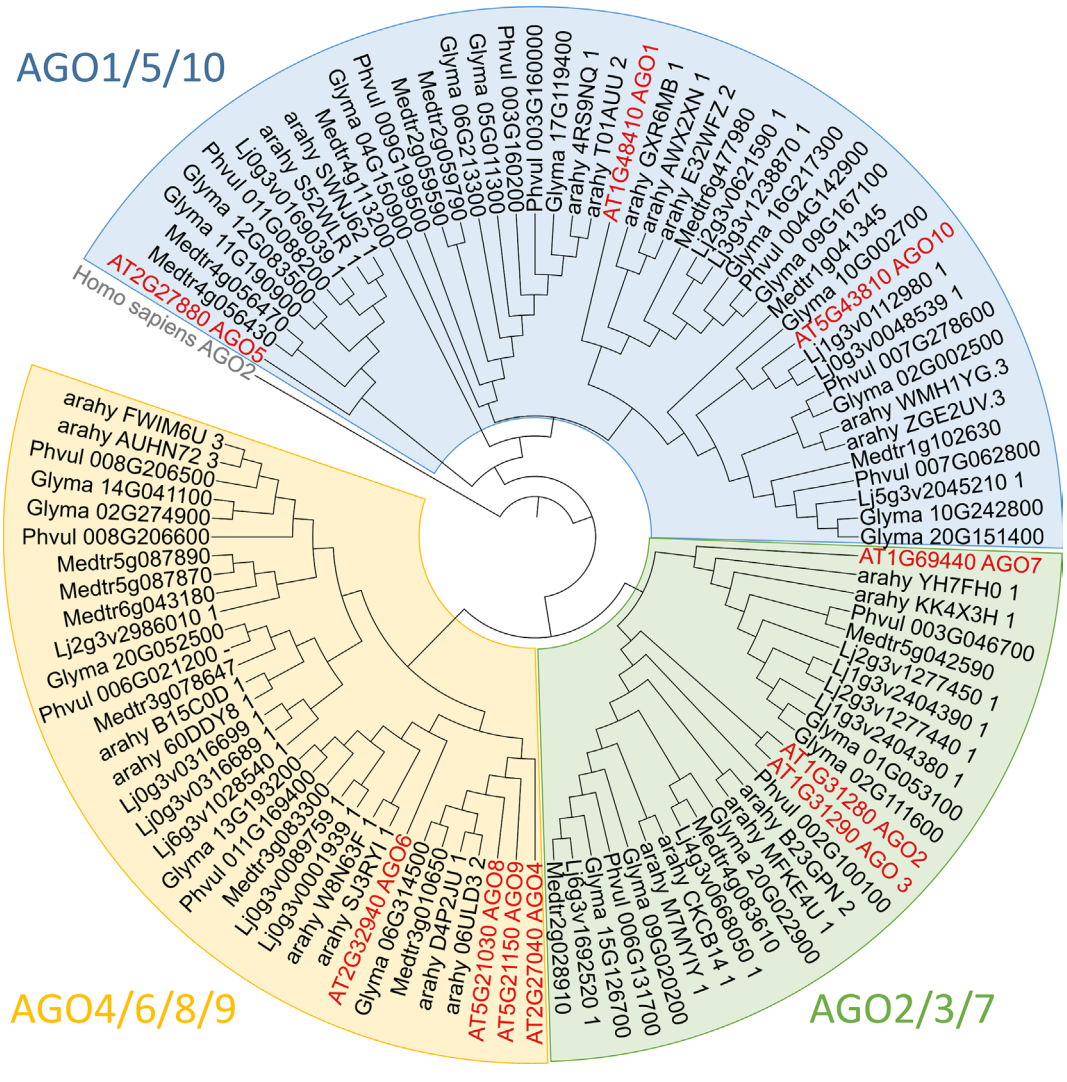

FIGURE 2 | Phylogenetic analysis of legume Argonaute (AGO) family proteins The protein sequences of selected AGOs were obtained from JGI Phytozome v. 12.1.6 (https://phytozome.jgi.doe.gov), Lotus Base (https://lotus.au.dk), and PeanutBase (https://peanutbase.org) and aligned using MAFFT online service v7.427 (Katoh et al., 2017) with FFT-NS-i option set. The phylogenetic tree was constructed using the average linkage (UPGMA) method and designed thanks to iTOL 4.4.2. Abbreviations for selected species are as follows: Medtr, *Medicago truncatula*; Lj, *Lotus japonicus*; Glyma, *Glycine max*; Phvul, *Phaseolus vulgaris*; arahy, *Arachis hypgaea*; AT, *Arabidopsis thaliana*.

the first member of the AGO family that has been demonstrated as a regulator of LRS (Reyero-Saavedra et al., 2017).

AGO10 may also be implicated in the regulation of LRS. It has been reported that AGO10 is capable of sequestering small RNAs, which consequently are not able to associate with their usual corresponding AGO family member (Zhu et al., 2011). This mechanism is involved in regulating the shoot apical meristem (SAM) in *Arabidopsis* (Zhou et al., 2015). To promote SAM differentiation, the action of miR166/165 on their target, which encodes the HD-ZIP III transcription factor, must be suppressed. To achieve this control, plants have selected a regulation system based on the sequestration of miR165/166 by AGO10, which has a higher affinity for these miRNAs than AGO1 and can promote their degradation (Yu et al., 2017). Although this mechanism has not been demonstrated directly in root apical meristem differentiation, some evidence suggests that it could be involved (Ma et al., 2017). In addition, the AGO10 regulatory mechanism is considered an ancient and ubiquitous process in land plant organ development. In *M. truncatula*, the miR166/*HD-ZIP III* node regulates both lateral root and nodule formation through the control of the apical region (Boualem et al., 2008). If the regulation of miR166/*HD-ZIP III* node by AGO10 proteins is a general mechanism, it is tempting to speculate that nodule development could also be controlled in this way. In support of this possible role in LRS, transcripts of *AGO10* group member genes in *M. truncatula*, *Glycine max,* and *P. vulgaris* are upregulated in nodules compared to root tissues (Phytozome v. 12.1.6). This reinforces the hypothesis that AGO10 could be a player in the regulation of nodule development.

AGO2/3/7 Clade

Beyond the phylogenetic grouping, members of the *AGO2/3/7* clade seem to be connected by an involvement in plant defense, employing different regulation mechanisms (Zhang et al., 2011; Fang and Qi, 2016; Rodríguez-Leal et al., 2016). Because AGO2 and AGO3 members are difficult to distinguish in legumes, due to the phylogenetically clustering of the two members by species of origin and not by member type (Zhang et al., 2015), here we focus on the "AGO2/3" group and AGO7 (**Figure 2**).

In *A. thaliana*, AGO2 is a key player in both antiviral defense and antibacterial immune response (Zhang et al., 2011; Carbonell and Carrington, 2015). Moreover, AGO2 is the only member of the *A. thaliana* AGO family reported as highly induced during *Pseudomonas syringae* infection (Zhang et al., 2011). AGO2 acts in this process by loading miR393b*, which targets transcripts of the gene *MEMB12* encoding a Golgi-localized, SDS-resistant, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), and then modulates the exocytosis of antimicrobial Pathogenesis-Related (PR) proteins. LRS is intimately linked to plant immunity (Toth and Stacey, 2015), and PR proteins seem to regulate the rhizobial infection process in soybean and *L. japonicus* (Bartsev et al., 2004; Hayashi et al., 2014). In this context, the involvement of AGO2/3 in the regulation of LRS should be considered. Supporting this hypothesis, AGO2/3 homologs in *M. truncatula*, *G. max,* and *P. vulgaris* are upregulated in nodules compared to root tissues (Phytozome v. 12.1.6). In addition, analysis of legumes AGO proteins shows that AGO2 has undergone gene duplication in *M. truncatula*, *G. max*, and *L. japonicus* (Bustos-Sanmamed et al., 2013) (**Figure 2**). This gene duplication of AGO2/3 suggests that the AGO2/3 isoforms may have diverged in their biological function and could be involved in novel processes, including LRS regulation.

AGO3 is one of the least studied members of the AGO family in plants and, to date, poor information is available about its functionality. Minoia and collaborators (2014) revealed that AGO3 binds siRNAs derived from potato spindle tuber viroid and could be involved in the defense against this pathogen. Similarly, in a recent preprint, Jullien and collaborators (2018) suggest a role of AGO3 in antiviral defense based on its confinement to vascular structures and the fact that most plant viruses use the phloem for systemic infection. However, further analyses are needed to understand the role of this AGO member and confirm its role in plant antiviral response. At this time, the link between AGO3 and the plant–microorganism interaction is speculative.

AGO7 is involved in the biogenesis and actions of trans-acting small interference RNAs (tasiRNAs, also called phasiRNAs), which are plant-specific endogenous siRNAs derived from long double-stranded RNA, and participate in plant development (Adenot et al., 2006). AGO7 also plays a critical role in the regulation of both plant immunity and antiviral defense (Adenot et al., 2006; Carbonell and

TABLE 2 | MiRNAs and Argonaute (AGO) proteins participating in different stages of the legume–rhizobia symbiosis (LRS)

miRNA	Associated AGO protein	Target Gene	Function in LRS	Legume Species	Reference
miR172c	AGO1/5	*AP2-1; NNC1*	Root hair deformation	*Phaseolus vulgaris; Glycine max*	Wang et al., 2014; Nova-Franco et al., 2015; Reyero-Saavedra et al., 2017
miR171c	AGO1	*NSP2*	Rhizobial infection	*Lotus japonicus*	De Luis et al., 2012
miR397	AGO1	*Laccase-Like*	Rhizobial infection	*Lotus japonicus*	De Luis et al., 2012
miR390/tasiARF	AGO7	*ARF3/4*	Rhizobial infection	*Medicago truncatula*	Allen et al., 2005
miR160	AGO1	*ARF10/16/17*	Auxins level	*Glycine max*	Turner et al., 2013; Nizampatnam et al., 2015
miR166	AGO1/10	*HD-ZIPIII*	Nodule development	*Medicago truncatula*	Boualem et al., 2008
miR167	AGO1/5	*ARF8*	Nodule development	*Phaseolus vulgaris; Glycine max*	Reyero-Saavedra et al., 2017
miR169	AGO1	*NF-YA1 (HAP2-1)*	Nodule development	*Medicago truncatula*	Combier et al., 2006

Carrigton, 2015). For example, AGO7 is also essential for the generation of the bacteria-induced small RNAs called long small interfering RNAs (lsiRNAs) (Katiyar-Agarwal et al., 2007). AtlsiRNA-1 is induced by bacterial pathogens and participates in plant resistance by silencing *AtRAP*, which encodes a RAP-domain protein involved in plant defense (Katiyar-Agarwal et al., 2007). This regulatory role of AGO7 in pathogen response mechanisms could be modulated to contribute to the fine-tuning of plant bacterial resistance under LRS. In support of this, *P. vulgaris* AGO7 is upregulated upon inoculation with rhizobia deficient in the production of NFs or lipopolysaccharides (Dalla Via et al., 2015), which are symbiotic signals able to suppress the plant defense response during symbiosis (Albus et al., 2001; Scheidle et al., 2004). Besides, mutation of the *AtAGO7* homolog in *L. japonicus* and *M. truncatula* reduces rhizobial infection and nodule number compared to the corresponding wild type (Li et al., 2014; Hobecker et al., 2017). Part of this response is also possibly due to the capacity of AGO7 to generate secondary small RNAs derived from the miR390-induced degradation of the *TAS3* transcript (Allen et al., 2005). The derived tasiRNAs target the *ARF2, 3* and *4* gene transcripts. These ARF TFs control part of the auxin signaling pathway, which also plays a key role in LRS (Breakspear et al., 2014).

AGO4/6/8/9 Clade

The AGO4/6/8/9 protein clade is oriented toward transcriptional regulation by DNA methylation (Mallory and Vaucheret, 2010; Duan et al., 2015). In legumes, this clade differs from other families. In *G. max*, *L. japonicus*, *M. truncatula*, and *P. vulgaris*, AGO4 and 6 are present but not AGO8. In the case of AGO9, this protein is absent in most legumes, except in *G. max*. This loss of diversity for the AGO8/9 group in legumes is compensated by the diversification of AGO4, which displays between two and four isoforms in the genome of model legumes (Bustos-Sanmamed et al., 2013) (**Figure 2**). This specific legume pool of AGO4 isoforms is phylogenetically separated from nonlegume AGO4, suggesting specialization in legumes. Supporting this hypothesis, in *G. max, M. trucatula*, and *P. vulgaris,* at least one of the AGO4 isoforms is differentially accumulated in nodules compared to root tissues, which suggests that this AGO4 isoform might play a role in the LRS (Phytozome v. 12.1.6).

DOES RHIZOBIAL SYMBIOSIS CAUSE DAMAGE IN THE LEGUME DNA?

Several studies have reported that plant pathogens can trigger damage in the host plant DNA (e.g., DNA double-strand breaks) (Song and Bent, 2014; Hadwiger and Tanaka, 2017). Some pathogen-induced DNA damage is triggered by reactive oxygen species (ROS) (Song and Bent, 2014; Hadwiger and Tanaka, 2017). It has been demonstrated that AGO2 and AGO9 play roles in DNA repair in *A. thaliana* (Wei et al., 2012; Oliver et al., 2014). Very recently, it has been reported that *Rhizobium huautlense* produces ROS in *Caenorhabditis elegans* intestinal cells, which then leads to DNA damage (Kniazeva and Ruvkun, 2019). Interestingly, during the rhizobial infection process, the production of ROS is essential for the formation of the infection thread (Damiani et al., 2016). Despite the evidence from animal cells and the fact that symbiotic rhizobia trigger ROS production, there is no experimental evidence to suggest that rhizobial symbiosis causes DNA damage in legume hosts. However, to allow rhizobial infection of the host, nodule cells undergo genome endoreduplication, often considered a protective mechanism against DNA damage to maintain whole-genome integrity (Maroti and Kondorosi, 2014). Further investigation is needed to explore whether rhizobia can cause DNA damage in legume hosts and whether AGO proteins (i.e., AGO2 and AGO9) participate in DNA repair in the context of LRS.

PERSPECTIVES AND CONCLUSIONS

Based on the participation of many different types of sRNAs, it is clear that different members of the AGO protein family might play crucial roles in LRS (**Figure 1**). However, it is still unclear how the participation of each AGO protein occurs and how it is regulated. Hence, the new challenge will be to understand how, when, and where AGO proteins are regulated during LRS. Having this knowledge will help us develop a clear idea about the relevance of AGO proteins in rhizobial symbiosis.

AUTHOR CONTRIBUTIONS

OV-L and DF designed the concept and organization of the manuscript. OV-L and DF wrote the manuscript with the help of MI-A, MR-S, TF-G, and MS-C.

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PLANT & CELL PHYSIOLOGY

A Novel Positive Regulator of the Early Stages of Root Nodule Symbiosis Identified by Phosphoproteomics

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Signals and signaling pathways underlying the symbiosis between legumes and rhizobia have been studied extensively over the past decades. In a previous phosphoproteomic study on the *Medicago truncatula–Sinorhizobium meliloti* symbiosis, we identified plant proteins that are differentially phosphorylated upon the perception of rhizobial signals, called Nod factors. In this study, we provide experimental evidence that one of these proteins, Early Phosphorylated Protein 1 (EPP1), is required for the initiation of this symbiosis. Upon inoculation with rhizobia, *MtEPP1* expression was induced in curled root hairs. Down-regulation of *MtEPP1* in *M. truncatula* roots almost abolished calcium spiking, reduced the expression of essential symbiosis-related genes (*MtNIN*, *MtNF-YB1*, *MtERN1* and *MtENOD40*) and strongly decreased nodule development. Phylogenetic analyses revealed that orthologs of MtEPP1 are present in legumes and specifically in plant species able to host arbuscular mycorrhizal fungi, suggesting a possible role in this association too. Short chitin oligomers induced the phosphorylation of MtEPP1 like Nod factors. However, the down-regulation of *MtEPP1* affected the colonization of *M. truncatula* roots by arbuscular mycorrhizal fungi only moderately. Altogether, these findings indicate that *MtEPP1* is essential for the establishment of the legume–rhizobia symbiosis but might plays a limited role in the arbuscular mycorrhizal symbiosis.

Keywords: Calcium spiking • Legume • Nodulation • Protein phosphorylation • Symbiosis.

Introduction

Legumes can fulfill a significant part of their nutrient needs through symbiotic associations with nitrogen-fixing rhizobia and arbuscular mycorrhizal (AM) fungi (Venkateshwaran et al. 2013, Castro-Guerrero et al. 2016). These symbioses are initiated by a signal exchange between the beneficial microbes and their legume partners. In the symbiosis between legumes and rhizobia (hereafter referred to as root nodule symbiosis), the interaction begins with the detection by the rhizobia of flavonoids and isoflavonoids produced by legume roots (Peters et al. 1986, Liu and Murray 2016). In turn, rhizobia produce diffusible 'Nod factors' (NF) that are lipo-chitooligosaccharides with specific chemical decorations (Dénarié et al. 1996). The legume host perceives NF via the LysM-domain receptor kinases NF Perception (NFP), LysM domain Receptor-like Kinase 3 (LYK3) and probably by an additional epidermal LysM receptor (Amor et al. 2003, Limpens et al. 2003, Arrighi et al. 2006, Murakami et al. 2018). A similar dialog between plants and AM fungi also involves the detection of a mixture of chitooligosaccharides, including lipo-chitooligosaccharides, which are produced by AM fungi (Genre et al. 2013, Sun et al. 2015, Luginbuehl and Oldroyd 2017). The mixture of chitooligosaccharides produced by AM fungi constitute the so-called 'mycorrhization factors' (MF) (Maillet et al. 2011). Upon perception of NF or MF, the transcription and phosphorylation of several symbiosis-related genes and proteins are activated, respectively. These molecular responses are crucial for triggering cellular rearrangements allowing the accommodation of either rhizobia or AM fungi in legume roots (Venkateshwaran et al. 2013, Luginbuehl and Oldroyd 2017).

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Despite differences in host range and cellular processes, these two endosymbioses share striking similarities. Genetic studies in the model legumes *Medicago truncatula* and *Lotus japonicus* have allowed the characterization of a 'common symbiosis pathway' (CSP) that is required for the establishment of symbiotic associations with both rhizobia and AM fungi (Lace and Ott 2018, Wang et al. 2018). Among the components of the CSP in *M. truncatula*, the leucine-rich repeat (LRR) receptor-like kinase, Does not Make Infections 2 (DMI2), which is localized at the plasma membrane, is assumed to participate in a receptor complex to perceive both NF and MF (Singh and Parniske 2012, Venkateshwaran et al. 2013, Luginbuehl and Oldroyd 2017). Another subset of proteins participating in the CSP includes different types of ion channels, such as the cation channel DMI1 localized at the nuclear envelope, calcium channels of the CNGC15 family, the calcium pump MCA8 and nucleoporins (NUP85, NUP133 and NENA). This set of ion channels is required to generate rapid oscillations in the nuclear and perinuclear calcium concentrations known as calcium spiking (Ané et al. 2004, Kanamori et al. 2006, Peiter et al. 2007, Saito et al. 2007, Groth et al. 2010, Charpentier et al. 2016). We demonstrated that mevalonate production is necessary and sufficient to activate calcium spiking in *M. truncatula* roots in response to rhizobial and AM fungal signals (Venkateshwaran et al. 2015). These calcium signatures are decoded by a nuclear calcium/calmodulin-dependent protein kinase (CCaMK/DMI3) that further transduces the signal by phosphorylating the transcription factor IPD3/CYCLOPS (Lévy et al. 2004, Miller et al. 2013, Singh et al. 2014). Downstream, different symbiosis-related transcription factors, such as Nodulation Signaling Pathway1 (NSP1)/NSP2, Nodule Inception (NIN), Ethylene Response Factor Required for Nodulation1 (ERN1) and Nuclear Factor YA-1 (NF-YA1)/(NF-YB1), are activated. These transcription factors act in a co-ordinated fashion to regulate the expression of a variety of genes required for the symbiosis with rhizobia and, for some of them, AM fungi (Genre and Russo 2016). Substantial evidence indicates that mutations in any of these core genes impair the signal transduction leading to the abortion of the symbiosis with both rhizobia and AM fungi.

It is widely accepted that CSP is a critical module for initiating both rhizobia and AM symbiosis (Wang et al. 2018). Recent studies have identified a variety of genes that are probably essential components of both rhizobial and AM symbioses. Hence, it has been suggested that those genes might be considered as new members of the CSP. Unfortunately, after detailed analysis, it has been demonstrated that several mutants of the proposed new members of the CSP are still able to establish symbiosis with one or both symbionts (reviewed in Lace and Ott 2018). However, it has also been proposed that genes falling in the first scenario (able still to interact with one of the symbionts) could be classified as members of an auxiliary pathway providing specificity for a particular symbiont (Lace and Ott 2018).

Although over the past two decades, considerable progress has been made to understand the early responses that control the establishment of the symbioses with rhizobia and AM fungi, many components of the signaling cascade controlling these symbioses remain unknown. For instance, the mechanisms of signal transduction from the plasma membrane to the nucleus remain unclear. Given the involvement of several active kinases (e.g. LYK3, DMI2 and DMI3) in this pathway, phosphorylation events are expected to be critical regulators in responses to symbiotic signals. In an attempt to identify new regulators, we previously performed a quantitative phosphoproteomic analysis in *M. truncatula* roots treated with purified NF from *Sinorhizobium meliloti* (Rose et al. 2012). Through this analysis, we identified 66 differentially phosphorylated proteins. Rapid phosphorylation of these proteins suggests their involvement in the early steps of this symbiosis. Unfortunately, the majority of these phosphorylated proteins have not yet been functionally characterized.

In this study, we characterized in detail one of these proteins whose phosphorylation levels increased consistently in response to purified NF in *M. truncatula* (Rose et al. 2012), hereafter termed Early Phosphorylated Protein1 (MtEPP1). An expression profile analysis by quantitative real-time PCR (qRT-PCR) revealed that *MtEPP1* is preferentially expressed in roots and nodules. Further expression analyses indicated that upon rhizobial inoculation, *MtEPP1* is expressed in curled root hairs. Down-regulation of the *MtEPP1* gene in *M. truncatula* transgenic roots almost abolished the NF-triggered calcium spiking, interfered with the expression of critical symbiosis-related genes (*MtNIN*, *MtNF-YB1*, *MtERN1* and *MtENOD40*) and severely decreased nodule development. Phylogenetic and synteny analyses indicated that *MtEPP1* is conserved in most land plants, even present in charophyte algae, but absent in the genome of non-host plants for AM fungi (e.g. *Arabidopsis thaliana*). This evolutionary pattern was similar to that of other genes involved in AM symbiosis (Delaux et al. 2014, Delaux et al. 2015). Despite this characteristic and that CO4 (*N*,*N*',*N*'',*N*'''-tetraacetyl chitotetraose) also triggered the phosphorylation of MtEPP1, the down-regulation of *MtEPP1* did not severely affect the root colonization by AM fungi. The data presented here indicate that *MtEPP1* is an essential component for the establishment of the root nodule symbiosis, but more dispensable for the symbiosis with AM fungi.

Results

MtEPP1 is a cytoplasmic protein and preferentially expressed in roots and nodules of *M. truncatula*

We previously reported that 66 proteins were differentially phosphorylated upon a 1 h treatment with purified NF (Rose et al. 2012). Among them, MtEPP1 (Medtr3g099200) was one of the most highly and consistently phosphorylated proteins (2.7-fold change), on Ser77. The *MtEPP1* gene contains two introns and three exons (**Fig. 1A**). It encodes a predicted protein of 41.6 kDa with an α-helix, a β-strand and four disordered regions (**Fig. 1B;** Supplementary Fig. S1). Additionally, MtEPP1 protein contains: (i) a putative pyridoxal phosphate-dependent transferase (IPR015421) domain that is tentatively located between amino

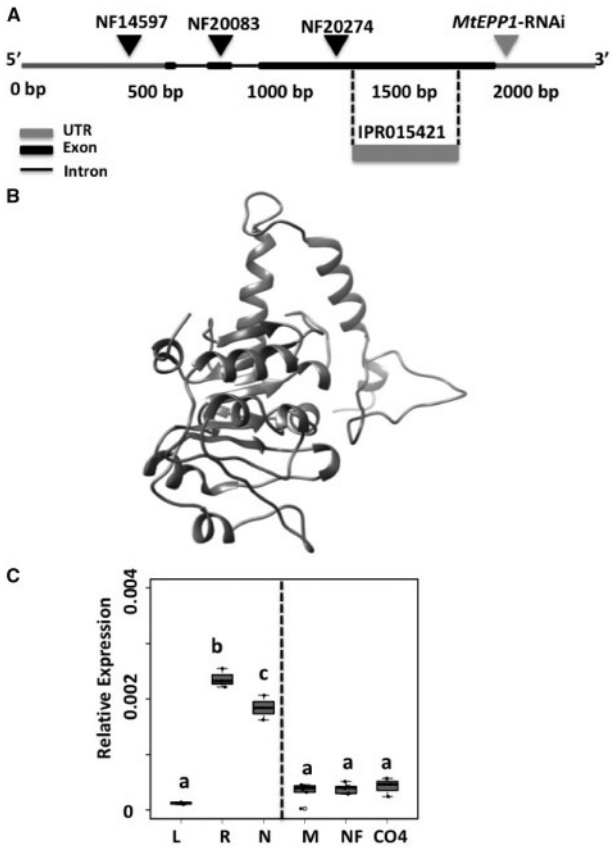

Fig. 1 Predicted gene and protein structures of MtEPP1 and its expression profile. (A) *MtEPP1* gene structure: untranslated regions (UTRs) are indicated in gray boxes, exons are indicated in black boxes, whereas solid dark lines indicate introns. The 3′-UTR used for the *MtEPP1*-RNAi construct is indicated by a gray arrow, whereas black arrows indicate the location of *Tnt-1* insertions identified in three independent *M. truncatula Tnt1*-insertion mutant lines (NF14597 at 491 bp, NF20083 at 795 bp and NF20274 at 1275 bp). Broken lines show the location of the putative pyridoxal phosphate-dependent transferase (IPR015421) domain. (B) Three-dimensional structure prediction of the MtEPP1 protein. This prediction was obtained by analyzing the primary structure in different protein structure prediction programs (e.g. Robetta Full-chain protein structure prediction server; http://www.robetta.org/submit.jsp). (C) Expression pattern of *MtEPP1* in leaves (L), roots (R), nodules (N), mock-inoculated- (M), 10^{-8} M NF-treated- or 10^{-7} M CO4-treated roots from 5 d old plants. Box plots represent the first and third quartile (horizontal box sides), and the minimum and maximum (outside whiskers). Data shown were obtained from four independent biological replicates. One-way ANOVA followed by a Tukey honest significant difference (HSD) test was performed (*P*-value <0.01). Statistical classes sharing a letter are not significantly different.

acids 233 and 333 and is highly conserved among legumes (Supplementary Fig. S2); (ii) one serine/threonine-rich region located between amino acids 128 and 150; and (iii) five potential protein-binding sites.

Analysis of the MtEPP1 protein sequence using protein localization prediction programs, such as SignalP 4.1 (Petersen et al. 2011), TMHMM 2.0 server (http://www.cbs.dtu.dk/services/TMHMM/) and PREDICT PROTEIN server (https://www.predictprotein.org) indicated the absence of signal peptide or potential transmembrane helices, suggesting that MtEPP1 protein is soluble and cytoplasmic. To corroborate this in silico prediction, the *MtEPP1* coding region was fused to enhanced green fluorescent protein (eGFP) in the C-terminus under the transcriptional regulation of the 35S promoter and expressed in transgenic *M. truncatula* roots by infection with *Agrobacterium rhizogenes* (**Fig. 2**) or in *Nicotiana benthamiana* leaves by agroinfiltration with *Agrobacterium tumefaciens* (Supplementary Fig. S3). Transgenic roots or leaves expressing non-fused eGFP were used as a control. As expected, the non-fused eGFP was observed in both the cytoplasm and nuclei of *M. truncatula* root cells and *N. benthamiana* leaf cells (**Fig. 2**; Supplementary Fig. S3). Green fluorescence (MtEPP1::eGFP) was detected in the cytoplasm of root cells, root hairs and leaf cells, confirming the predictions of the cytoplasmic localization of MtEPP1.

To gain further insight into the expression profile of *MtEPP1*, we performed a data-mining analysis on transcriptional data from *M. truncatula* available in the *M. truncatula* Transcriptome Atlas (https://mtgea.noble.org/v3/; Benedito et al. 2008). Based on available transcription data in *M. truncatula*, *MtEPP1* shows high expression in roots, roots hairs and nodules, and a weak expression in leaves and flowers. To confirm this observation, we evaluated the expression of *MtEPP1* in leaves, roots, and nodules from *M. truncatula* plants by qRT-PCR. This expression analysis by qRT-PCR confirmed the preferential expression of *MtEPP1* in roots and nodules (**Fig. 1C**). Altogether, these data indicate that *MtEPP1* is preferentially expressed in roots and nodules and that its protein is located in the cytoplasm.

The expression of *MtEPP1* is induced in root hairs upon NF treatment

The fact that MtEPP1 was rapidly phosphorylated upon a 1 h treatment with purified NF, and that its gene is preferentially expressed in roots and nodules, prompted us to evaluate whether these symbiotic signals regulate the expression of MtEPP1. To test this hypothesis, we assessed the expression of *MtEPP1* in roots of the wild-type genotype Jemalong A17 treated for 1 h with NF. This expression analysis revealed no significant differences in *MtEPP1* expression in response to the NF within this time frame (**Fig. 1C**).

Given that specific genes can be expressed at particular cell types, assessing their expression in whole organs (e.g. roots) might result in dilution or even loss of signal. Recently, it was reported that Medtr3g099200, which encodes MtEPP1, is one of the *M. truncatula* genes displaying expression in root hairs (Damiani et al. 2016). Additionally, in the same study, it was reported that the expression of Medtr3g099200 was slightly (fold change of 1.5) induced after 20 h of treatment with purified NF (Damiani et al. 2016). Based on this evidence, we evaluated the spatiotemporal activity of the *MtEPP1* promoter in response to purified NF. We cloned a 2 kb fragment of the

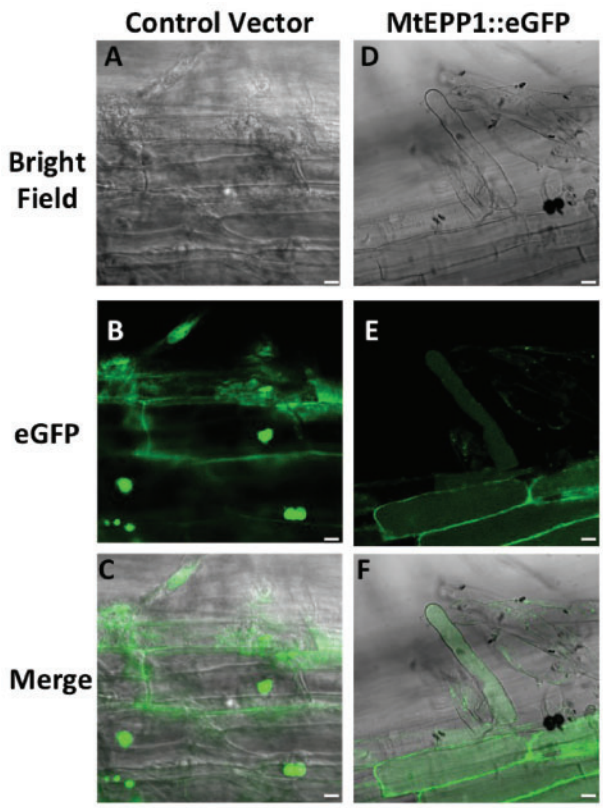

Fig. 2 MtEPP1 localizes at the cytoplasm. (A–C) The eGFP fluorescence in *M. truncatula* root cells expressing the control vector (35S::eGFP). (D–F) The cytoplasmic localization of the MtEPP1::eGFP fusion under control of the 35S promoter in *M. truncatula* root cells. Scale bars represent 10 µm. Pictures shown are representative of three biological replicates, each one containing five transgenic roots.

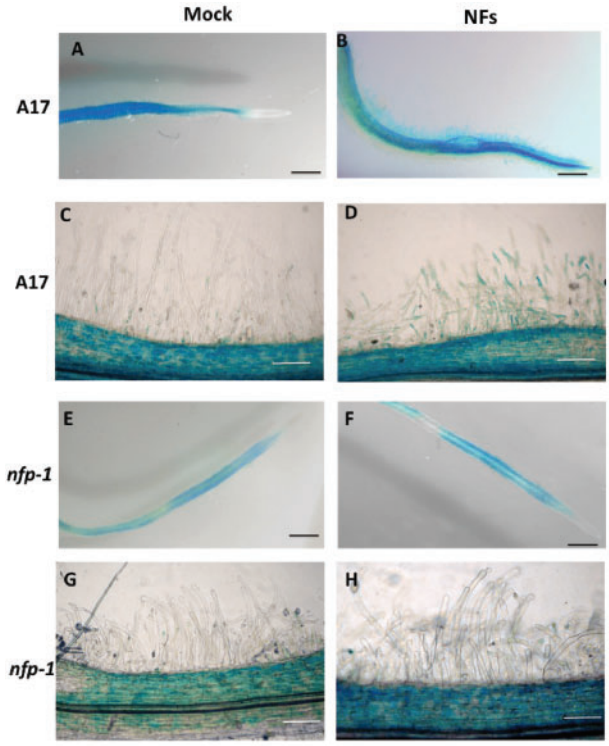

Fig. 3 *MtEPP1* expression is induced in the root hairs in response to NF. Transgenic roots from Jemalong A17 (A–D) and the *nfp-1* (E–H) mutant plant expressing the *pMtEPP1::GUS* construct were treated for 1 h with water (mock) or 10 nM NF. (A, B) and (E, F) The *pMtEPP1::GUS* activity in whole roots from Jemalong A17 and *nfp-1* mutant plants, respectively. (C–D) and (G–H) The *pMtEPP1::GUS* activity in root hairs of roots from Jemalong A17 and *nfp-1* mutant plants, respectively. Both mock- and NF-treated transgenic roots were stained for 1 h at 37°C. Scale bars represent 500 µm for (A), (B), (E) and (F), and 200 µm for (C), (D), (G) and (H). Images shown are representative of five biological replicates, each one containing 10 transgenic roots.

MtEPP1 promoter (*pMtEPP1*) that was immediately upstream of the *MtEPP1* translation initiation codon and generated transcriptional fusion to the *GUS* (β-glucuronidase) coding sequence. The *pMtEPP1::GUS* construct was transfected into *M. truncatula* through the *A. rhizogenes*-mediated transformation method, and transgenic roots were treated with NF for 1 h. We observed GUS activity in the entire root (**Fig. 3A**), with some faint staining in the root hairs of mock-inoculated transgenic roots (**Fig. 3C**). In contrast, we observed strong staining in the root hairs from NF-inoculated transgenic roots (**Fig. 3B, D**). This spatiotemporal activity was absent in root hairs from NF-inoculated transgenic roots generated in the *M. truncatula nfp-1* mutant (**Fig. 3E–H**). Altogether, these data indicate that *MtEPP1* expression is up-regulated in root hairs upon NF recognition.

The expression of *MtEPP1* is induced in rhizobia-induced curled root hairs

Because we observed a definite increase in the activity of the *MtEPP1* promoter in the root hairs upon NF treatment, and as rhizobia colonize the legume roots through the infection thread, we investigated whether the activity of the *MtEPP1* promoter is located at specific sites of the root hairs in response to rhizobia. Transgenic roots generated in the wild-type genotype Jemalong A17 and expressing the transcriptional fusion *pMtEPP1::GUS* were inoculated with *S. meliloti*. At 3 d post-inoculation, we observed a substantial activity of the *MtEPP1* promoter in curled root hairs (**Fig. 4**). These data indicate that *MtEPP1* expression is induced in rhizobia-induced curled root hairs.

MtEPP1 is required for root nodulation

Because NF triggered MtEPP1 phosphorylation and NF and rhizobia induce the expression of its gene in the root hair, we thus hypothesized that *MtEPP1* might play a role in the establishment of the symbiosis between *M. truncatula* and *S. meliloti*. To test this hypothesis genetically, we searched for mutants in the *M. truncatula Tnt1*-insertion mutant collection of the Noble Research Institute (Tadege et al. 2008) and identified three independent *Tnt1*-insertion lines, NF20083, NF14597 and NF20274, and heterozygous mutants were obtained (**Fig. 1A**). Despite many attempts and various experimental conditions tested, we

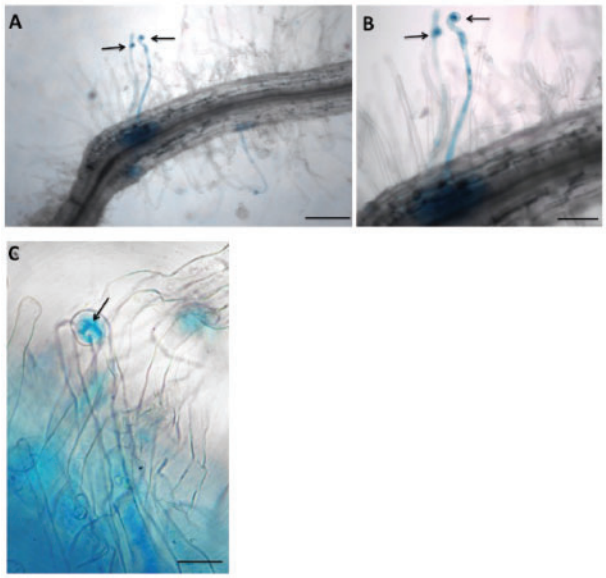

Fig. 4 *MtEPP1* expression is induced in rhizobia-induced curled root hairs. Transgenic roots from Jemalong A17 expressing the *pMtEPP1::GUS* construct were inoculated with *S. meliloti* 1021. Three-days post-inoculation, transgenic roots showing DsRED fluorescence were collected and stained for 1 h at 37°C. *pMtEPP1* activity was detected in curled root hairs (A, B). *pMtEPP1* activity was also detected at the curl of the infected root hair (C). Scale bars represent 250 µm (A), 100 µm (B) and 50 µm (C). Pictures shown are representative of five biological replicates, each one containing 10 transgenic roots.

have never been able to recover any homozygous mutant in the progeny of these heterozygous plants. Indeed, heterozygous plants showed similar expression levels of *MtEPP1* as well as no significant differences in the number of nodules per plant. In the absence of homozygous mutants, we generated an RNA interference (RNAi) construct targeting *MtEPP1* (*MtEPP1*-RNAi) and used *A. rhizogenes*-mediated transformation to silence *MtEPP1* in the roots of *M. truncatula*. The expression of *MtEPP1* in *M. truncatula* transgenic roots expressing the RNAi construct was reduced on average by 70% compared with the roots transformed with a control vector (Supplementary Fig. S4). Additionally, the expression of the three homologs of *MtEPP1* was not affected in transgenic roots expressing the *MtEPP1*-RNAi construct, confirming the specificity of this construct (Supplementary Fig. S4). We observed that transgenic roots expressing the *MtEPP1*-RNAi construct were shorter compared with the transgenic roots expressing the control vector (Supplementary Fig. S5A). In spite of this reduction in the root growth, no apparent difference in the morphology of the *MtEPP1*-RNAi root hairs was observed (Supplementary Fig. S5B, C). To test whether the reduction in the expression of *MtEPP1* affected the establishment of the symbiosis with *S. meliloti*, we performed nodulation assays on *M. truncatula* transgenic roots. These nodulation assays indicated that 85% ($n = 200$) of the control vector transgenics developed on average four nodules per plant. In contrast, 10% ($n = 190$) of the *MtEPP1*-RNAi transgenic roots developed one nodule per plant, whereas the other 90% of the *MtEPP1*-RNAi composite plants did not develop any nodules at all (**Fig. 5A, B**). These results demonstrate that down-regulation of *MtEPP1* negatively and strongly affects nodule development.

MtEPP1 is required for NF-induced root hair branching and the activation of symbiotic gene expression

To test whether *MtEPP1* is necessary to activate early responses of the legume–rhizobia symbiosis, transgenic roots expressing either *MtEPP1*-RNAi or control vector were treated with 10^{-8} M purified NF. Sixteen hours after treatment, 90% ($n = 100$) of the control vector transgenic roots showed characteristic NF-induced root hair branching, whereas only 13% ($n = 150$) of *MtEPP1*-RNAi-transformed roots did (**Fig. 5C, D;** Supplementary Fig. S5D, E).

The fact that *MtEPP1* is required to induce NF-induced root hair branching and nodule formation suggests that *MtEPP1* is likely to be an essential component participating in the early responses of the legume–rhizobia symbiosis. To investigate further the role played by *MtEPP1*, we monitored the expression of *MtENOD11*, in response to NF. We used *M. truncatula* Jemalong A17 seedlings stably transformed with the promoter of *MtENOD11* fused to the coding sequence of *GUS*, *pMtENOD11-gusA* (Journet et al. 2001). *Agrobacterium rhizogenes*-mediated root transformation was performed on these seedlings to silence *MtEPP1*. GUS activity was determined in roots after treatment with NF. Upon 16 h of NF treatment, we observed a substantial GUS activity in transgenic roots transformed with the control vector (**Fig. 6A**). In contrast, we saw only a weak GUS activity in *MtEPP1*-silenced transgenic roots. To validate this *MtENOD11* expression data further, we evaluated its expression by qRT-PCR along with four additional symbiosis-related genes: *MtNIN*, *MtERN1*, *MtNF-YB1* and *MtENOD40*. As expected, NF did not induce the expression of *MtENOD11* in *MtEPP1*-silenced transgenic roots. In contrast, we observed a 1,000-fold change in control vector transgenic roots (**Fig. 6B**). Similar results were found when the expression of *MtNIN*, *MtERN1* and *MtNF-YB1* was analyzed, which were not induced upon NF treatment in *MtEPP1*-silenced transgenic roots (**Fig. 6C, E**). Although we detected an induction (2-fold change) in the expression of *ENOD40* in transgenic roots expressing *MtEPP1*-RNAi, its expression level was much lower than the response observed in the transgenic control roots (**Fig. 6F**). Together, these results indicate that *MtEPP1* is required to activate early events of the legume–rhizobia symbiosis.

MtEPP1 is required for NF-induced calcium spiking

A characteristic response to NF is the induction of nuclear and perinuclear calcium spiking that controls the activation of early nodulin gene expression (Ehrhardt et al. 1996, Wais et al. 2002). Because we observed a reduction in the expression of selected early nodulin genes (**Fig. 6**), we hypothesized that the down-regulation of *MtEPP1* might affect the NF-induced calcium

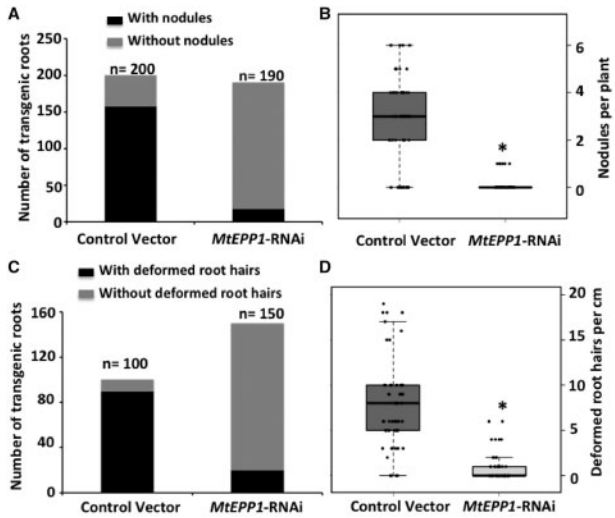

Fig. 5 Down-regulation of *MtEPP1* negatively affects nodule formation. (A) Number of transgenic roots showing nodules or NF-induced branched root hairs (C). (B) Number of nodules per plant. (D) Number of NF-induced deformed root hairs per centimeter of roots. Box plots represent the first and third quartile (horizontal box sides), and the minimum and maximum (outside whiskers). An asterisk indicates a significant difference according to one-way ANOVA (*P*-value <0.001). Data shown were obtained from 10 biological replicates each one containing 20 different composite *M. truncatula* plants.

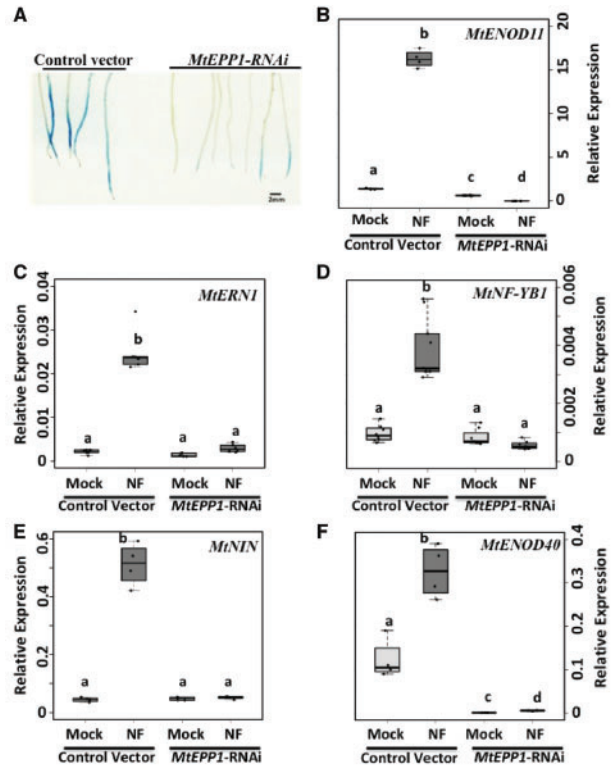

Fig. 6 Down-regulation of *MtEPP1* reduces the activation of early nodulin genes. (A) Down-regulation of *MtEPP1* strongly decreased the *MtENOD11*-induced expression as revealed by p*MtENOD11-gusA* histochemical assay and (B) by qRT-PCR in response to NF. NF-triggered expression of *MtERN1* (C), *MtNF-YB1* (D), *MtNIN* (E) and *MtENOD40* (F) was almost abolished in *MtEPP1*-silenced transgenic roots. Box plots represent the first and third quartile (horizontal box sides), and the minimum and maximum (outside whiskers). Data shown were obtained from four independent biological replicates. One-way ANOVA followed by a Tukey honest significant difference (HSD) test was performed (*P*-value <0.01). Statistical classes sharing a letter are not significantly different.

spiking. To test this hypothesis, we analyzed the calcium spiking triggered by NF in transgenic roots of *M. truncatula* expressing the calcium sensor, Yellow Chameleon 3.6 (YC3.6), along with either the *MtEPP1*-RNAi construct or control vector. We observed NF-induced calcium spiking in 75% ($n = 15$) of the analyzed control vector transgenic roots (**Fig. 7A, B**). In contrast, only 6% ($n = 16$) of *MtEPP1*-RNAi transgenic roots showed an irregular calcium spiking (lower intensity and less frequent irregular calcium spikes) (**Fig. 7A, B**). These results indicate that *MtEPP1* is required to induce the calcium spiking triggered by NF.

MtEPP1 is present in legumes and host plants of arbuscular mycorrhizal fungi

Because the expression the *MtNIN* and *MtENOD11* genes as well as the activation of the calcium spiking are required to establish symbiosis with both rhizobia and AM fungi (Kosuta et al. 2003, Boisson-Dernier et al. 2005, Guillotin et al. 2016), and the fact that the down-regulation of the *MtEPP1* gene almost abolished these molecular responses, we hypothesized that MtEPP1 might also participate in the establishment of the symbiosis with AM fungi. To test this hypothesis, we first investigated whether MtEPP1 is present in the genome of host plants of AM fungi. To this aim, we constructed a single phylogeny analysis with available genomic and transcriptomic data. This analysis revealed the existence of potential orthologs of *MtEPP1* in different legumes. We also found orthologs of *MtEPP1* in those plants that can establish symbiosis with AM fungi, including liverworts (Supplementary Fig. S6A). In contrast, we did not find any homolog in the genomes of *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Aethionema arabicum*, *Brassica rapa*, *Capsella rubella*, *Thellungiella halophila* (Brassicaceae), *Utricularia gibba* (Lentibulariaceae), *Beta vulgaris* (Amaranthaceae), *Nelumbo nucifera* (Nelumbonaceae), *Pinus taeda*, *Picea abies* (Gymnosperms, Pinaceae) and the moss *Physcomitrella patens* that are all non-host plants for either rhizobia or AM fungi (Khade et al. 2010, Delaux et al. 2014). To confirm the absence of *EPP1* in non-host species, we conducted a synteny analysis on the surrounding region of *MtEPP1* loci in *M. truncatula*, *Amborella trichopoda* (basal angiosperm), *Gossypium raimondii* (Malvales), *Carica papaya* (Brassicales) and the corresponding genomic block in the non-host *A. thaliana* Col-0, *B. rapa*, *A. arabicum*, sugar beet and *N. nucifera*. Whereas these blocks are well conserved between AM fungi host species, *EPP1* is missing in the genome of all five non-host species (Supplementary Fig. S6B). Our data indicate that *EPP1* is conserved in legumes and host plants of AM fungi, and might also be essential to interact with this symbiont.

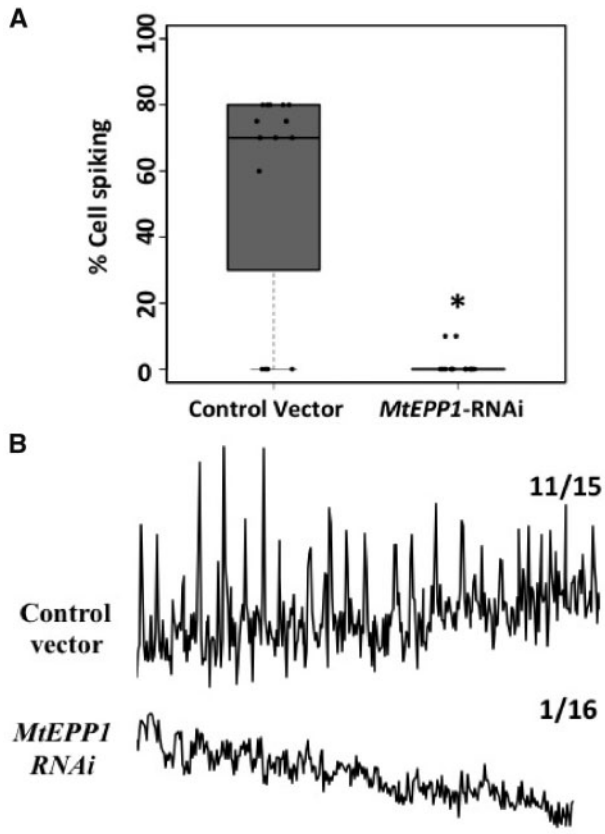

Fig. 7 Down-regulation of *MtEPP1* abolishes the NF-triggered calcium spiking. (A) The percentage of cells showing spiking was significantly higher in control vector-transformed roots than in *MtEPP1*-RNAi-transformed roots. An asterisk indicates a significant difference according to Fisher's exact test (*P*-value <0.001). (B) Representative calcium traces in roots transformed with control vector or *MtEPP1*-RNAi recorded over 25 min using the YC3.6 reporter.

Symbiotic signals of AM fungi induce the phosphorylation of MtEPP1

MtEPP1 was initially selected because of its significant phosphorylation on its Ser77 in response to NF (Rose et al. 2012). However, our phylogenetic analysis indicates the existence of MtEPP1 ortholog genes in the genome of host plants of AM fungi. Additionally, we found that the Ser77 residue is conserved in the EPP1 orthologs of different AM host plants (Supplementary Fig. S6C). With this evidence, we hypothesized that AM fungi symbiotic signals might also trigger the phosphorylation of MtEPP1. To determine whether phosphorylation of Ser77 of MtEPP1 is also induced by CO4, molecules produced by AM fungi, we used a multiple reaction monitoring approach. This targeted liquid chromatography–tandem mass spectrometry (LC-MS/MS) approach allows the quantifiction of a given analyte, a phosphopeptide in this case, using several *m/z* coming from the specific fragmentation of this analyte. Combined with a synthetic internal standard, this very sensitive approach allows accurate quantification of particular phosphosites. Total proteins were extracted from *M. truncatula* roots treated with CO4 or mock treated for 24 h. These samples were then used to determine the change in phosphorylation state of Ser77 on MtEPP1. Whereas synthesized standards are detected at a similar level in both cases, the amount of Ser77 phosphorylated peptides was significantly higher (~30-fold change) in CO4-treated samples compared with mock-treated samples (Supplementary Fig. S7A, B). These results indicate that CO4 also triggers MtEPP1 phosphorylation.

To evaluate whether CO4 induces the expression of *MtEPP1*, roots from the wild-type Jemalong A17 genotype were treated with this AM fungi-derived molecule, and the expression of MtEPP1 was assessed by qRT-PCR. This expression analysis showed that CO4, like NF, did not induce the expression of *MtEPP1* (**Fig. 1C**). Since *MtEPP1* is expressed in the root hairs in response to purified NF, we evaluated the spatiotemporal activity of the *pMtEPP1* promoter in response to CO4 by using the transcriptional fusion *pMtEPP1*::*GUS* expressed in *M. truncatula* transgenic roots (Supplementary Fig. S8). This spatiotemporal expression assay indicated that *MtEPP1* is expressed in the root hairs upon treatment with CO4 (Supplementary Fig. S8). In summary, the *MtEPP1* expression is up-regulated in root hairs, and its protein is phosphorylated upon CO4 treatment.

MtEPP1 plays a limited role in the establishment of the arbuscular mycorrhizal symbiosis

Finally, since *MtEPP1* is essential to activate early symbiotic events and was found only in AM host plants, we hypothesized that *MtEPP1* might be required for successful colonization by AM fungi. To test the potential role of *MtEPP1* in AM symbiosis, we conducted mycorrhization assays on roots transformed either with the *MtEPP1*-RNAi construct or with the control vector. Six weeks post-inoculation, transgenic roots were screened for AM colonization. Silencing of *MtEPP1* barely affected the symbiosis with AM fungi, which was reflected in a reduction by 10% of the density of arbuscules and other fungal structures (e.g. hyphopodia and vesicles) (**Fig. 8**). This result indicates that *MtEPP1* plays a limited role in the AM symbiosis.

Discussion

Over the past two decades, remarkable progress has been made in understanding the genetic mechanisms underlying the establishment of the root nodule symbiosis. For instance, a CSP controlling the molecular dialog with both rhizobia and AM fungi has been identified as a result of this intensive effort (Venkateshwaran et al. 2013, Genre and Russo 2016). Recent large-scale analyses on the early events occurring during the establishment of the root nodule symbiosis have uncovered the existence of potential new regulators. For instance, phosphoproteomic and transcriptomic analyses on *M. truncatula* roots treated for 1 h with purified NF enabled us to identify a significant number of differentially regulated genes and phosphorylated proteins (Rose et al. 2012). Similarly, Damiani et al. (2016) identified a set of genes that were differentially regulated at the root hairs upon 4 h treatment with purified NF. Nevertheless, few of these identified genes or phosphorylated proteins have been functionally characterized yet.

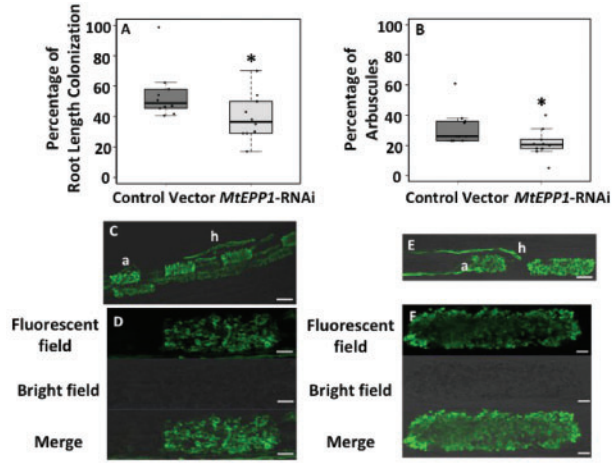

Fig. 8 MtEPP1 plays a limited role in the AM fungi symbiosis. (A) Percentage of root length colonization that includes arbuscules, hyphopodia and spores, and (B) percentage of arbuscules in *MtEPP1*-silenced or control vector transgenic roots. Data shown were obtained from four independent biological replicates, each one containing 10 transgenic roots. An asterisk indicates a significant difference according to Fisher's exact test (*P*-value <0.05). Arbuscules (a) and hyphopodia (h) which developed in control vector transgenic roots (C and D) were similar to those formed in MtEPP1-silenced roots (E and F). The scale bar represents 10 μm in (C) and (E), and 2 μm in (D) and (F) Pictures shown are representative of four biological replicates, each one containing 10 transgenic roots.

In this study, we provide evidence supporting the role of MtEPP1, one of the phosphorylated proteins identified in our previous phosphoproteomic analysis (Rose et al. 2012), in the molecular dialogue between *M. truncatula* and *S. meliloti*. In this study, we demonstrated that the expression of *MtEPP1* is induced in curled root hairs in response to *S. meliloti* inoculation. Further experimentation on *MtEPP1*-silenced *M. truncatula* roots revealed that the activation of the NF-triggered calcium spiking, the expression of critical early nodulin genes (*MtNIN*, *MtNF-YB*, *MtERN1*, *MtENOD11* and *MtENOD40*) and the NF-induced root hair branching were almost abolished. Accordingly, we showed that only 10% of the analyzed *MtEPP1*-silenced transgenic roots were able to develop just one nodule. Additionally, we showed that orthologous genes of *MtEPP1* are only present in legumes and the host plants of AM fungi. Likewise, we demonstrated that CO4 also triggered the MtEPP1 phosphorylation. Nevertheless, the down-regulation of *MtEPP1* did not lead to a dramatic reduction of the root colonization by AM fungi. These results led us to propose that MtEPP1 is an essential component for *M. truncatula* to communicate with rhizobia efficiently, but more dispensable to establish symbiosis with AM fungi. Alternatively, the presence of two paralogs of *MtEPP1* in *M. truncatula* might result in genetic redundancy masking a potentially more pronounced AM phenotype.

Growing evidence indicates that the expression of genes controlling early events of the root nodule symbiosis is induced at the root hairs upon NF detection (Haney et al. 2011, Breakspear et al. 2014, Damiani et al. 2016, Arthikala et al. 2017). For instance, very recently it was reported that the *MtNFH1* gene, encoding an NF hydrolase participating in the regulation of the NF levels, is expressed in curled root hairs in response to rhizobia (Cai et al. 2018). Similarly, Arthikala et al. (2017) reported that the *PvRbohA* gene, which is involved in rhizobia-triggered reactive oxygen species production, is expressed in the root hairs and in the growing infection thread. In this study, we found a similar spatiotemporal expression pattern for *MtEPP1*, which was detected in rhizobia-induced curled root hairs. These expression data indicate that *MtEPP1* is a novel component required to activate early responses in the root nodule symbiosis.

The activation of the nuclear/perinuclear calcium spiking and its subsequent decoding by DMI3 is crucial for activating the transcriptional activator IPD3/CYCLOPS, which, in turn, enables the expression of the transcription factor genes *NSP1*/*NSP2*, *NIN*, *NF-YA/B* and *ERN1* (Venkateshwaran et al. 2013, Genre and Russo 2016). These transcription factor genes are required to activate the expression of different symbiosis-related genes participating in root hair curling, infection chamber formation and entrapping of rhizobia (Cerri et al. 2012, Soyano et al. 2013, Singh et al. 2014, Lace and Ott 2018). It has been demonstrated that *dmi1*, *dmi2*, *cngc15s*, *nena*, *nup85* and *nup133* mutant plants are not able to activate calcium spiking in response to NF (Wais et al. 2000, Kanamori et al. 2006, Saito et al. 2007, Charpentier et al. 2016). Additionally, we have reported that mevalonate can trigger calcium spiking, probably through the activation of DMI1 (Venkateshwaran et al. 2015). Here, we demonstrated that the down-regulation of *MtEPP1* almost abolished the NF-induced calcium spiking. Accordingly, we showed that the expression of *MtNIN*, *MtNF-YA*, *MtERN1*, *MtENOD11* and *MtENOD40*, as well as the NF-triggered root hair branching, was dramatically reduced in *MtEPP1*-silenced roots. These data indicate that MtEPP1 is an essential element required to activate calcium spiking in response to rhizobia. Likewise, with these data it is also tempting to speculate that MtEPP1 might indirectly participate in the activation of genes coding for enzymes involved in mevalonate biosynthesis, for instance 3-hydroxy-3-methylglutaryl-CoA reductase1 (HMGR1).

It is widely demonstrated that NF and MF can activate the same core of genes and induce similar calcium signatures (Wang et al. 2018). However, it is still unclear how similar early molecular responses lead to the establishment of two different symbioses. Along this line, it has been proposed that CSP, along with the calcium spiking, is an essential module required to initiate symbiotic responses, but NF and MF might activate an independent auxiliary pathway providing specificity (Lace and Ott 2018). Evidence supporting this hypothesis has been presented through the overexpression of the NF receptors, which led to the activation of specific responses of the root nodule symbiosis (Reid et al. 2014). Here, we provide evidence indicating that MtEPP1 is conserved in legumes and AM fungi host plants, a hallmark of genes participating in the establishment of the symbiosis with AM fungi. Likewise, we demonstrated that CO4 also triggered the phosphorylation of MtEPP1. Despite this evidence, the down-regulation of MtEPP1 reduced the root colonization by AM fungi by 10%. Altogether,

these data suggest that MtEPP1 plays a limited role in the symbiosis with AM fungi. Likewise, these data also provide evidence supporting the existence of an auxiliary pathway that provides specificity to rhizobia; however, further research is needed.

Conclusions

In conclusion, the data presented in this study led us to conclude that *MtEPP1* is a novel component essential to trigger calcium spiking leading to the activation of early nodulin genes. The fact that the down-regulation of *MtEPP1* did not compromise the symbiosis with AM prompted the suggestion that this gene might be dispensable to establish this endosymbiosis; however, further investigation is required to prove this hypothesis.

Materials and Methods

Plant material

Medicago truncatula line Jemalong A17, the R108 line, the transgenic line Jemalong A17 *pMtENOD1-gusA* (Journet et al. 2001), three *Tnt1*-insertion mutant lines of *MtEPP1* (NF20083, NF14597 and NF20274) derived from R108, as well as *nfp-1* (Amor et al. 2003), mutants derived from Jemalong A17, were used in this study. Seeds were scarified and surface-sterilized. Seeds were plated on 1% deionized water agar plates supplemented with 1 μM GA_3. Seeds were subsequently vernalized for precisely 48 h at 4°C and were then germinated by incubating them at room temperature overnight. On the next day, seedlings with equal root length were transferred into plates containing modified Fahräeus medium (Catoira et al. 2000) supplemented with 0.1 μM aminoethoxyvinylglycine (AVG) (Sigma-Aldrich). Fahräeus medium plates were incubated in a growth chamber at 21°C and 16 h of light.

Bacterial strains and culture conditions

The control vector pH7GWIWG2(II)-YC3.6 was propagated in *Escherichia coli* DB 3.1 cells, whereas the hairpin RNAi construct against *MtEPP1* (see below for details), the transcriptional fusion *MtEPP1*::GUS and the translational fusion MtEPP1::eGFP were propagated in *E. coli* DH5α cells. *Escherichia coli* cells were handled using standard procedures.

The *A. rhizogenes* MSU440 strain was used to induce transgenic roots in *M. truncatula* plants (see below for details), whereas the *A. tumefaciens* GV3101 strain was used to transfect *N. benthamiana* leaves. *Agrobacterium rhizogenes* and *A. tumefaciens* cells were grown on 5 mg l^{-1} tryptone/3 mg l^{-1} yeast extract, 6 mM $CaCl_2$ (TY) plates for 2 d at 30°C; 50 μg ml^{-1} spectinomycin or 50 μg ml^{-1} kanamycin was added to select for the presence of plasmid vectors.

Sinorhizobium meliloti strain 1021 was used to inoculate *M. truncatula* plants. The *S. meliloti* cells were grown on TY plates supplemented with 50 μg ml^{-1} streptomycin for 2 d at 30°C.

Mutant screening

To identify homozygous mutant lines of *MtEPP1*, we obtained the mutant lines NF20083, NF14597 and NF20274 from the Samuel Roberts Noble Foundation *Tnt1*-insertion mutant collection that carried a *Tnt1* insertion in the coding sequence of *MtEPP1*. Genomic DNA from 200 individual plants (from each insertion line) was isolated by using the GeneCatch Plant Genomic DNA Purification Kit (Epoch Life Sciences). We used the gene-specific primer (5′-GCTTCAGCTATGATG TGAGCTGG-3′) and the *Tnt1*-specific primer (5′-AGTTGGCTACCAATCCAACAA GGA-3′) to identify homozygous *Tnt1*-insertion mutants by PCR.

Plasmid construction

To analyze the activity of the *MtEPP1* promoter, a 2,073 bp DNA fragment upstream of the start codon was PCR-amplified from genomic DNA of *M. truncatula* A17 Jemalong by using specific primers. To generate the *pMtEPP1*::*GUS* construct, we used the Golden Gate cloning strategy as described by Servin-Pujol et al. (2017). The resulting *pMtEPP1*::GUS cassette was then cloned into the binary vector pCAMBIA_CR1 containing the constitutively expressed DsRED protein (Servin-Pujol et al. 2017).

To analyze the subcellular localization of MtEPP1, the full *MtEPP1* CDS (coding sequence) without a stop codon was amplified. The resulting PCR product was then cloned into the pENTR-D-TOPO (Thermo Fisher Scientific) vector. The resulting pENTR-*MtEPP1* plasmid was recombined into the pK7FWG2-RR binary vector containing the open CDS of eGFP, yielding the C-terminal MtEPP1::eGFP fusion.

To generate an RNAi construct against *MtEPP1*, a specific fragment of 350 bp from the C-terminal end of *MtEPP1* was amplified using gene-specific primers. The amplified fragment was then cloned into the pENTR-D-TOPO (Thermo Fisher Scientific) vector. The resulting pENTR-*MtEPP1-RNAi* plasmid was recombined into the pH7GWIWG2(II)-YC3.6 binary vector containing the constitutively expressed fluorescent YC3.6 protein (Riely et al. 2011). The correct orientation was verified by PCR using the primers Forward-Intron (5′-GCA CACCAGAGCATATATATTGGTGG-3′) and Reverse-35SPromoter (5′-CCACTA TCCTTCGCAAGACCCTTCC-3′).

All constructs were verified by DNA sequencing. Primer sequences for plasmid constructions are shown in Supplementary Table S1.

Agrobacterium rhizogenes-mediated transformation

Binary vectors with *pMtEPP1*::*GUS*, MtEPP1::eGFP or *MtEPP1*-RNAi constructs were mobilized into *A. rhizogenes* MSU440 by electroporation. The empty vectors pH7GWIWG2(II)-YC3.6 or pK7FWG2-RR were used as controls. *Agrobacterium rhizogenes*-mediated transformation was performed according to Boisson-Dernier et al. (2001). Composite plants (plants with the transformed root system and untransformed shoot system) were grown in nitrogen-free Fahräeus medium plates under the environmental conditions described above. YC3.6 or DsRED fluorescence in the transgenic roots was observed with a fluorescence stereomicroscope.

Transfection of *Nicotiana benthamiana* leaves

Transient expression of the MtEPP1::eGFP fusion protein was conducted in *N. benthamiana* for subcellular localization of MtEPP1. Briefly, the abaxial epidermis of young leaves of 4-week-old *N. benthamiana* plants was infiltrated with *A. tumefaciens* GV3101 suspension harboring either MtEPP1::eGFP or the control vector construct. At 3 d post-infiltration, the infiltrated leaf areas were cut and analyzed under an LSM 510 Meta confocal microscope. The eGFP was excited at 489 nm, and the fluorescence emission was collected at 509 nm.

MtEPP1 promoter activity in response to NF and *S. meliloti*

To evaluate the *MtEPP1* promoter activity, *A. rhizogenes*-mediated *pMtEPP1*::*GUS* transgenic roots were generated in the *M. truncatula* wild-type line Jemalong A17 as well as in the *nfp-1* mutant plant. Composite plants were grown in Fahräeus medium plates under the environmental conditions described above. Roots were treated with 10 nM NF or inoculated with *S. meliloti* 1021. Upon 1 h NF treatment or 3 d post-rhizobia inoculation, transgenic roots showing DsRED fluorescence were immersed in GUS staining solution [0.05% 5-bromo-4-chloro-3-indolxyl-β-D-glucuronic acid, 100 mM sodium phosphate buffer (pH 7), 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM Na_2EDTA and 0.1% Triton X-100] and incubated for 1 h at 37°C. After being cleared in diluted NaClO and rinsing in phosphate buffer, roots were examined under bright-field microscopy. For these experiments, five biological replicates, each one with 10 composite plants, were included.

Histochemical assays for p*MtENOD11*::*GUS*

To analyze the expression of the symbiotic gene *MtENOD11* by histochemical staining, *A. rhizogenes*-mediated *MtEPP1*-RNAi transgenic roots were generated in *M. truncatula* plants stably transformed with a p*MtENOD11*::*GUS* construct. Composite plants were treated with 10 nM NF for 16 h. Transgenic roots showing YC3.6 fluorescence were immersed in the GUS staining solution and incubated for 2 h at 37°C. After clearing in diluted NaClO and rinsing in phosphate

buffer, roots were analyzed under a stereomicroscope and imaged using an EPSON V370 Photo scanner. For this experiment, five biological replicates, each one with 10 composite plants, were included.

Gene expression analysis

To analyze the expression of *MtENOD11*, *MtENOD40* and *MtNIN* genes, composite plants were treated with 10 nM NF or mock treated (control) for 16 h as described above. For *MtEPP1*, *MtERN1* and *MtNF-YB1*, composite plants were treated with 10 nM NF or mock treated for 1 h. NF-treated transgenic roots showing YC3.6 fluorescence were harvested, immediately frozen in liquid nitrogen and stored at −80°C until used. Total RNA was isolated from three NF-treated transgenic roots using the GeneCatch Plant RNA Purification kit (Epoch Life Sciences) following the manufacturer's instructions. cDNA was synthesized from 1 μg of genomic DNA-free total RNA. This cDNA was used to analyze the above-mentioned symbiosis-related genes by qRT-PCR in a Step-One qPCR thermocycler (Applied Biosystems). The housekeeping gene *MtActin* was used to normalize gene expression levels. The expression level of different genes was calculated according to the equation $E = P_{eff}^{(-\Delta Ct)}$. P_{eff} is the primer set efficiency computed using the LinRegPCR program (Ramakers et al. 2003), and ΔCt (cycle threshold) was calculated by subtracting the Ct value of the housekeeping gene from the Ct values of a given gene. The nucleotide sequences of the qRT-PCR primers used in this study are provided in Supplementary Table S1. For this experiment six biological replicates, each one with three technical replicates, were included.

Calcium spiking imaging and data analysis

To analyze the NF-induced calcium spiking, *M. truncatula* composite plants expressing the control vector pH7GWIWG2(II)-RNAi-YC3.6 (control) or the construct pH7GWIWG2(II)-*MtEPP1*-RNAi-YC3.6 were used. Composite plants with transgenic roots expressing the calcium sensor YC3.6 were selected based on strong yellow fluorescence under a fluorescence stereomicroscope. These composite plants (RNAi or control) were mounted on custom-made plastic slides using a modified Fahräeus medium to which 10 nM NF was added before calcium imaging. Calcium measurements were performed under a Zeiss LSM 510 Meta confocal microscope as reported by Riely et al. (2011). Briefly, young and emerging root hairs from the apical region of the root elongation zone were chosen for calcium measurements. The calcium sensor was excited at 458 nm using an argon laser. The cyan fluorescent protein (CFP; 473–505 nm) and Förster resonance energy transfer FRET; 536–546 nm) emissions were collected. For time-lapse analyses, images were obtained every 3.5 s. Background CFP and FRET signal intensities were subtracted. The FRET/CFP ratio was calculated and was plotted against time.

Root hair deformation analysis

Medicago truncatula composite plants expressing the construct *MtEPP1*-RNAi or control vector and growing in Fahräeus plates were treated with 10 nM purified NF from *S. meliloti*. Upon 16 h of treatment, transgenic roots showing YC3.6 fluorescence were observed with a bright-field microscope. For this experiment, 15 biological replicates, each one with 10 plants, were included.

Nodulation assay

Medicago truncatula composite plants expressing the construct *MtEPP1*-RNAi or control vector and growing in nitrogen-free Fahräeus medium plates were inoculated with 1 ml of *S. meliloti* 1021 with an OD_{600} value of 0.01. The lower part of the dishes was wrapped in aluminum foil to keep the roots in the dark. Plates were then placed vertically in a growth chamber at 24°C in 16 h light/8 h dark cycle conditions. At 14 d post-inoculation, the total number of nodules was counted in roots showing YC3.6 fluorescence exclusively. For this experiment, 10 biological replicates, each one containing 20 plants, were used.

In vivo phosphorylation of EPP1 by mass spectrometry

To test the effect of hitooligosaccharides on MtEPP1 phosphorylation, plants were grown as previously described and treated with 100 nM CO4 or mock treated (0.5% ethanol in water) for 1 h. Plants were then quickly harvested and flash-frozen in liquid nitrogen, and ground with a mortar and pestle for phosphoprotein extraction. Samples were further homogenized in grinding buffer supplemented with phosphatase inhibitors using sonication (1 cm probe, 4×30 s and 50% duty cycle) while kept on ice as described by Minkoff et al. (2014). The resulting supernatant was filtered through four layers of Miracloth (Calbiochem) and underwent centrifugation (16,000×*g*, 20 min and 4°C) to remove debris. Proteins were precipitated from the sample supernatant with acetone overnight at −20°C by adding 4 vols. of ice-cold acetone to 1 vol. of plant extract. The resulting precipitate was resuspended in 2% SDS, 1 mM dithiothreitol, Tris–HCl buffer before methanol/chloroform/water protein extraction. Methanol/chloroform extraction was performed by adding 4 vols. of methanol to 1 vol. of resuspended plant extract. The mixture was vortexed, and 1 vol. of chloroform was added before additional vortexing. Three volumes of water were added, and the solution was vortexed and subsequently centrifuged for 15 min (2,500×*g*, 20 °C). The top layer was removed and discarded via a serological pipette. Then, 3 vols. of methanol were added, and the solution was vortexed before centrifugation. Pellets were washed once with 80% acetone. Extracted protein was resuspended in 6 M urea, 1× phosSTOP (Roche) and quantified using a bicinchoninic acid assay kit. For each sample, 5 mg of protein was spiked with isotopically labeled phosphopeptide standard (WpSGPITP[+6]K), synthesized by the University of Wisconsin-Madison Biotechnology Center's peptide synthesis core facility. Samples were reduced with 5 mM dithiothreitol (30 min at 55°C) and alkylated using 15 mM iodoacetamide (30 min at room temperature). Samples were digested for 2 h at 37°C using LysC (Wako) at a 1:100 enzyme:protein ratio then diluted with 1.5 M urea using 20 mM Tris pH 8 and further digested with trypsin (Promega) at a 1:250 enzyme:protein ratio overnight at 37°C. Samples were acidified with 0.5% (v/v) trifluoroacertic acid (TFA) to stop enzymatic digestion and desalted using C-18 solid-phase extraction columns (Waters). Phosphopeptide enrichment was performed using homemade TiO_2 columns containing 4 mg of TiO_2 particles (10 μm; GLSciences), as described by Minkoff et al. (2014). LC-MS analysis was performed using the Eksigent NanoLCUltra 2 D system with the cHiPLC nanoflex microfluidic C18 column (75 mm, 120 Å) coupled to the AB SCIEX 5500 QTRAP mass spectrometer. Analytical separation was performed using a linear gradient of 0–30% acetonitrile with 0.1% formic acid over 70 min at a flow rate of 300 nl min^{-1}. Masses monitored are indicated in Supplementary Table S2. Peak areas were integrated using Skyline version 2.5.

Sequence collection and phylogenetic analysis

To collect sequences corresponding to potential MtEPP1 orthologs, the *MtEPP1* sequence was searched in genome and transcriptome data sets using BLASTp and tBLASTn, respectively. Hits with an E-value $<10^{-50}$ were selected for the phylogenetic analysis. Sequences used are indicated in Supplementary Table S3. Also, for non-host species, the best hits were reciprocally blasted on the *M. truncatula* genome to confirm the absence of orthologs. Collected sequences were aligned using MAFFT (http://mafft.cbrc.jp/alignment/server/) and the alignment was manually curated with BioEdit. Phylogenetic trees were generated by both Neighbor–Joining and the maximum likelihood algorithm in MEGA6. Partial gap deletion (95%) was used together with the JTT substitution model (Gamma 2 parameters) and the 'subtree pruning and regrafting' heuristic algorithm (SPR3). Bootstrap was calculated using 500 replicates.

Synteny analysis

Synteny analysis of the MtEPP1 locus in *M. truncatula*, *A. trichopoda*, *G. raimondii*, *C. papaya*, *A. thaliana* Col-0, *B. rapa*, *A. arabicum*, *B. vulgaris* and *N. nucifera* genomes was performed as described previously (Delaux et al. 2014). Briefly, 100 kb upstream and downstream of *MtEPP1* and the ortholog of *Carica papaya* were compared using COGE GEvo (https://genomevolution.org/CoGe/GEvo.pl). Syntenic genomic blocks in *A. thaliana* Col-0, *B. rapa*, *A. arabicum*, *B. vulgaris* and *N.nucifera* were identified by GoGe-BLAST and added to the analysis.

Arbuscular mycorrhization assays

For AM assays, 2-week-old *M. truncatula* composite plants were transferred to pots filled with clay-based soil (Turface, Profile®). Each container was inoculated

with 400 spores of *Rhizophagus irregularis* 197198 (PremierTech®). Plants were watered three times per week with a Long Ashton solution (Hewitt 1966) with a low phosphate concentration (10 μM) and with water when needed. After 6 weeks, transgenic roots showing YC3.6 fluorescence were harvested, stained with wheat germ agglutinin and conjugated to Alexa Fluor 488 (Invitrogen). Fungal structures (e.g. hyphopodia and arbuscules) were visualized under a Zeiss LSM 510 Meta confocal microscope as reported by Pumplin et al. (2010). For each root system, the numbers of hyphopodia and arbuscules were counted. The percentage of root length colonization was also calculated. Four independent replicates with at least seven plants each were used for this study.

Statistical analyses

All the statistical analyses were conducted using R software 3.0.1. The specific statistical tests performed are indicated in the legend of the corresponding figures.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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