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Los microRNAs como reguladores de la respuesta de frijol al estrés abiótico.

TESIS

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Abreviaturas

RNA: Ácido ribonucléico

DNA: Ácido desoxiribonucléico

Nasa: Enzima nitrogenasa

APX: Ascorbato peroxidasa

Al: Aluminio

ALMT1: Transportador de malato dependiente de Al

mRNA: RNA mensajero

Alt: Toxicidad por Aluminio

Cu: Cobre

Cut: Toxicidad por Cobre

miRNA: microRNA

Mn: Manganeso

Mnt: Toxicidad por Manganeso

CSD: Cu/Zn Superóxido dismutasa

RESUMEN

El frijol es una de las leguminosas más importante para consumo humano en el mundo, la cual establece una relación simbiótica con las bacterias fijadoras de nitrógeno del género rhizobia. El rhizobia infecta la raíz de su leguminosa hospedera, induce la formación de nódulos donde se establece y nitrógeno para proveerlo a la planta, lo cual reduce costos de fertilización.

La toxicidad por metales en el suelo es uno de los estreses abióticos que afecta la producción de cultivos de frijol y la fijación simbiótica de nitrógeno. Es importante conocer a profundidad cuáles son los reguladores involucrados en las respuestas de la planta para contender con el exceso de metales tales como aluminio (Al) y cobre (Cu) que son frecuentes en el tipo de suelos en los que crece el frijol.

En este trabajo caracterizamos las respuestas fisiológicas de las plantas de frijol inoculadas con *Rhizobioum tropici* CIAT899, expuestas a toxicidad por Al comparadas con las plantas en condiciones control. Entre las respuestas de la raíz y los nódulos destacaron la lipoperoxidación, la acumulación de especies reactivas de oxígeno, la acumulación de callosa y la muerte celular así como una disminución en la actividad de la enzima nitrogenasa en los nódulos.

Entre los reguladores relacionados con las respuestas ante estrés abiótico se han identificado a los microRNAs (micro Ácidos Ribonucleicos) como componentes primordiales en esta regulación, por lo cual en este trabajo analizamos la expresión diferencial de los miRNAs ante toxicidad por metales, utilizando el enfoque de macroarreglos de miRNAs. Identificamos 28 miRNAs de frijol que responden a la toxicidad por aluminio y 26 miRNAs a la toxicidad por cobre. Estos incluyen, los reguladores miR164, miR170 miR393, miR396, pvu-miR1511 que inducen su

expresión, así como los miR157, miR169 y miR398 que inhiben su expresión en la raíz y los nódulos de plantas de frijol expuestas a toxicidad por los metales Al y Cu. Además nos planteamos analizar la función del pvu-miR1511 y su blanco predicho (SP1L) que aún se desconoce, utilizando el enfoque de genética reversa. Observamos que las plantas compuestas que sobreexpresan el miR1511 mostraron una disminución en el tamaño de los nódulos así como en la actividad de la nitrogenasa, indicando que la función de este microRNA podría estar relacionada con el desarrollo de los nódulos.

Como parte de la caracterización de miRNAs responsivos a metales, analizamos la expresión de miR398 y de sus genes blanco CSD1 y Nod19 en raíces, nódulos y hojas de frijol comprobando el papel que tiene este miRNA en la homeostasis de Cu.

Los resultados de este trabajo proveen las bases para analizar y concluir sobre la función de miRNAs específicos en la regulación de la respuesta del frijol a la toxicidad por metales.

ABSTRACT

Commom bean is one of the most important legumes for human consumption in the world, which establishes a symbiotic relationship with the nitrogen fixing bacteria of the rhizobia genus. Rhizobia infects the root of its host legume, induces the formation of nodules where it is established and nitrogen to provide it to the plant, which reduces fertilization costs.

Metal toxicity in the soil is one of the abiotic stresses that affect the production of bean crops and symbiotic nitrogen fixation. It is important to know in depth which regulators are involved in the plant responses to cope with excess metals such as aluminum (Al) and copper (Cu) that are frequent in the type of soils in which beans grow

In this work we characterize the physiological responses of bean plants inoculated with Rhizobioum tropici CIAT899, exposed to Al toxicity compared to plants under control conditions. Among the root and nodule responses were lipoperoxidation, accumulation of reactive oxygen species, callus accumulation and cell death as well as a decrease in the activity of the enzyme nitrogenase in the nodules

Among regulators related to abiotic stress responses, microRNAs (micro Ribonucleic Acids) have been identified as primordial components in this regulation, so in this work we analyze the differential expression of miRNAs against metal toxicity, using the approach of miRNAs macroarray. We identified 28 bean miRNAs that respond to aluminum toxicity and 26 miRNAs to copper toxicity. These include the miR167, miR170 miR393, miR396, pvu-miR1511 regulators that induce their expression, as well as the miR157, miR169 and

miR398 regulators that inhibit their expression in bean plant nodules exposed to Al and Cu.

We also analyzed the role of pvu-miR1511 and its predicted target (SP1L), which is still unknown, using the reverse genetics approach. We observed that the composite plants that overexpress the miR1511 showed a decrease in the nodule size as well as in the activity of the nitrogenase, indicating that the function of this microRNA could be related to the nodule development.

As part of the characterization of metal responsive miRNAs, we analyzed the expression of miR398 and its target genes CSD1 and Nod19 in roots, nodules and leaves of bean, confirming the role of this miRNA in Cu homeostasis.

The results of this work provide the basis for analyzing and concluding about the role of specific miRNAs in the regulation of bean response to metal toxicity.

1. INTRODUCCIÓN

1.1 Las plantas leguminosas y la fijación biológica de nitrógeno.

La familia *Fabaceae* (leguminosas) es uno de los grupos taxonómicos con mayor número de plantas angiospermas contando con 18,000 especies, muchas de ellas importantes como alimento o forraje, por su alto contenido proteico (Lewis et al., 2005). Estas plantas tienen la capacidad para asociarse en simbiosis con bacterias de suelo de la familia *Rhizobiaceae* que fijan el nitrógeno atmosférico libre, el cual es asimilado por las plantas.

El proceso de la fijación biológica del nitrógeno es llevado a cabo por las bacterias rhizobia que proliferan en el suelo, las cuales reconocen y responden a compuestos secretados por las raíces de las plantas leguminosas, tales como flavonoides. Estas moléculas inducen a los genes *nod*, los cuales codifican para las proteínas requeridas para la síntesis y secreción de los factores Nod (Figura 1). Los factores Nod son lipo-quito-oligosacáridos con modificaciones químicas reconocidas por el hospedero. Los factores Nod inician los cambios en el desarrollo de la planta en el proceso de nodulación, incluyendo la deformación del pelo radicular, la despolarización de la membrana, las oscilaciones de calcio intracellar y la iniciación de la división celular en el cortex de la raíz, el cual establece un primordio y consigo la proliferación celular que resultará en la formación de un nuevo órgano: el nódulo. (Dénarié et al., 1996; Geurts et al., 2002) (Figura 1).

Durante las primeras fases de la simbiosis los rhizobia crecen, se dividen y migran a una nueva estructura tubular llamada hilo de infección que generalmente es iniciado cuando rhizobia queda atrapada entre dos paredes celulares de los pelos radiculares. La formación del hilo infectivo normalmente ocurre cuando un pelo radical deformado forma una curvatura o bucle, y la bacteria se une al pelo radical quedando atrapada entre las paredes celulares (Callaham et al. 1981). El hilo de

infección va migrando dentro del pelo radicular, dentro de la célula epidermal, mientras los rhizobia se dividen y crecen dentro de él, asegurando así la colonización de un suficiente número de células del nódulo (Van den Bosch K. et al., 1989; van Spronsen et al. 1994).

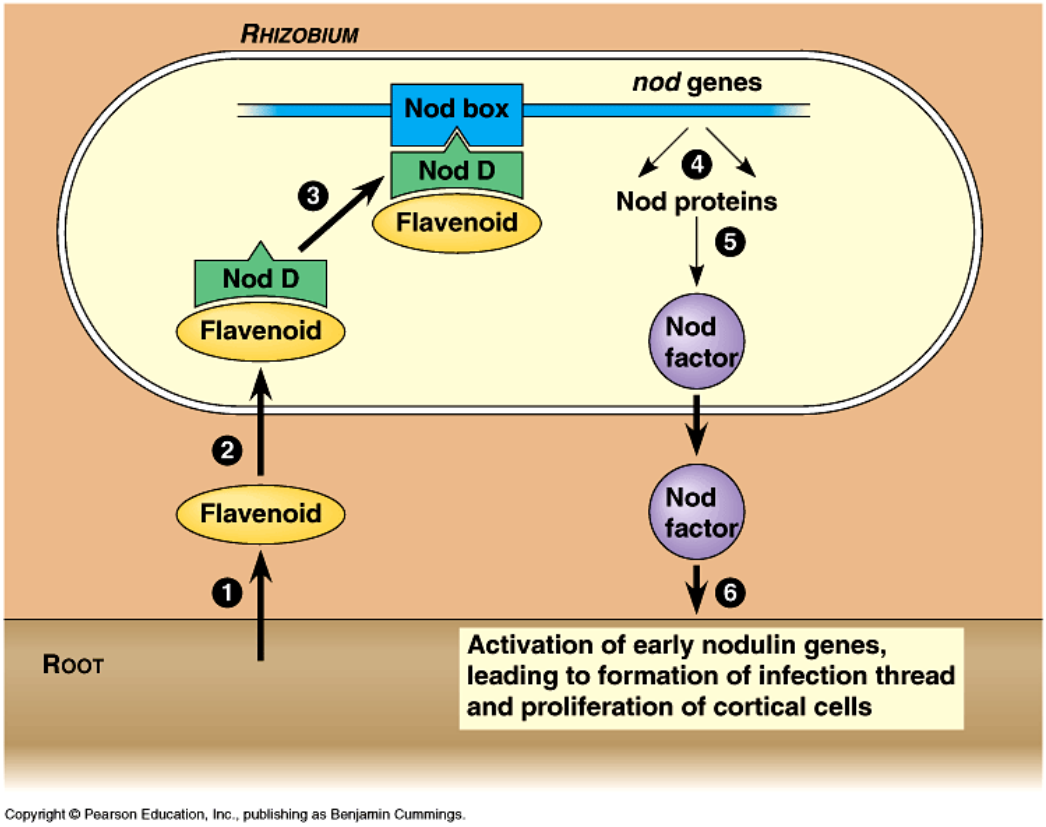

Figura 1. Pasos iniciales del proceso de infección por rhizobia en una raíz de una leguminosa.

Cuando ya se ha formado el nódulo, las bacterias llevan a cabo la fijación biológica del nitrógeno atmosférico, la cual consiste en la reducción del N_2 a amonio (NH_4+) por la enzima nitrogenasa (Nasa). Esta ruta metabólica es la segunda ruta metabólica más importante para el mantenimiento de la biósfera, después de la fotosíntesis (Sprent J.I 2009).

En las diferentes leguminosas podemos encontrar dos tipos de nódulos: determinados o indeterminados, lo cual se relaciona con el sitio en donde se inducen

las divisiones mitóticas en la raíz para su formación. Si las divisiones se dan en el córtex interno se originan nódulos indeterminados y si se dan en el córtex externo, nódulos determinados. Ambos tipos de nódulos presentan una estructura anatómica distinta. En el caso de los nódulos indeterminados rhizobium induce las primeras divisiones en el plano anticlinal originando el primordio del nódulo. Desde el inicio se establece una polaridad en el primordio manteniendo la actividad meristemática en el ápice, causando un crecimiento del primordio hacia el exterior, mientras que las capas celulares inferiores se van diferenciando (Foucher F. y Kondorosi E., 2000). En los nódulos determinados a diferencia de los indeterminados, no hay un meristemo permanente. Su crecimiento se basa en la expansión en vez de en la división celular, razón por la que presentan una morfología esférica en lugar de cilíndrica (Hirsch A.M., 1992). La causa de la ausencia de un meristemo permanente la podemos encontrar en el proceso de formación ya que las primeras divisiones celulares en respuesta a la presencia de *Rhizobium* son anticlinales y se producen en las células corticales en seguida se genera otro foco de división celular en el periciclo. Posteriormente, estos dos meristemos convergen generando el primordio nodular, en el cual podemos encontrarnos células no vacuoladas procedente de las divisiones de las células corticales conformando el tejido central del nódulo, y células con un elevado grado de vacuolización procedentes de las divisiones en el periciclo, componiendo el parénquima nodular que rodea al tejido central; gran parte de la actividad mitótica en la región central del nódulo se pierde transcurridos 12 a 18 días tras la inoculación (Newcomb W. et al. , 1979; Rolfe B.G. y Gresshoff P.M., 1988).

El frijol (*Phaseolus vulgaris*), forma nódulos de tipo determinado, en este órgano se convierten el N2 en amoniaco gracias a la actividad del complejo enzimático nitrogenasa (Nasa).

En los nódulos, el bacteroide convierte el N_2 en amoniaco gracias a la actividad del complejo enzimático (Nasa), el cual está constituido por dos metaloproteínas y puede contener tres tipos de grupos prostéticos (Sprent, 2009). La proteína I es llamada también dinitrogenasa o Fierromolibdeno-proteína. El otro componente, la proteína II, es nombrada dinitrogenasa reductasa o Fierro-proteína.

La proteína I es un tetrámero de alrededor de 220,000 Da, formado por dos tipos de subunidades α2 β2, de masa molecular semejante, y que son los productos de los genes *nifDK* . Esta proteína contiene el cofactor con fierro y molibdeno (FeMo-Co). La proteína también contiene dos grupos prostéticos diferentes: cuatro grupos 4Fe-4S, llamados grupos P ligados de manera covalente a residuos de cisteína de las subunidades α y β y los dos grupos FeMoCo unidos a la subunidad α, en donde son transportados los electrones y es reducido el N_2. (Leigh 2002; Sprent 2009)

La proteína II es un dímero de alrededor de 68,000 Da, formado por dos subunidades idénticas unidas por un grupo prostético único de 4Fe-4S. El gen responsable de la síntesis de esta proteína es *nifH*. Esta proteína tiene la función de transportar los electrones del donador fisiológico de electrones (ferrodoxina o flavodoxina) hacia la proteína I para llevar a cabo la reducción de la molécula de N_2. La Nasa es sumamente lábil a la inactivación por el 0_2 incluso para su purificación se requieren condiciones anaeróbicas estrictas debido a que el cofactor FeMoCo es irreversiblemente desnaturalizado y oxidado. La fijación biológica del nitrógeno es un proceso que requiere un gasto considerable de energía es por eso que está sometida a una estricta regulación (Leigh, 2002).

Como se mencionó anteriormente la Nasa genera el nitrógeno asimilable (NH4+), que es metabolizado en las células nodulares a amidas o ureidos, que luego son exportados vía xilema al resto de la planta, donde éstos son usados (Mayz, 1997). El primer producto de la reacción de fijación es NH3 (amoníaco), pero este es

rápidamente protonado, formándose NH4+, tomando parte en las reacciones de asimilación (Sprent y Sprent, 1990).

El amonio es un inhibidor de la síntesis de la nitrogenasa, por lo que es necesaria su rápida asimilación; en el citosol de las células infectadas, éste es procesado por la enzima glutamina sintetasa formándose glutamina, la cual puede ser exportada o usada para restaurar el ácido glutámico (Sprent y Sprent, 1990; Ortega et al., 2004).

Las leguminosas simbióticas pueden ser separadas en dos grupos de acuerdo a los productos exportados desde los nódulos: las exportadoras de amidas (asparagina, glutamina) y las exportadoras de ureidos (alantoína y ácido alantoico).

El primer grupo incluye varias especies de regiones templadas, entre estas *Lupinus subcarnosus*, *Pisum sativum* y *Medicago sativa* y el segundo varias especies tropicales, como *Glycine max*, *Phaseolus vulgaris* y *Vigna unguiculata* (Miflin y Habash, 2002; Harrison et al., 2003).

El proceso de fijación de nitrógeno puede ser afectado por múltiples factores abióticos, uno de los más estudiados y que se ha demostrado que tiene un importante impacto es el estrés hídrico, ya que se ha señalado que éste provoca una limitación de carbono en los nódulos de las leguminosas que podría ser la causa del descenso de la fijación de nitrógeno en las mismas (Gálvez et al. 2005).La salinidad y la toxicidad por metales como Cadmio también provoca estrés oxidativo el cual se ha relacionado también como un factor importante en la disfunción del proceso de fijación de nitrógeno, debido a la sobreproducción de ROS (Becana et al. 2010; Marino et al. 2013).

1.2 El cultivo del frijol y el estrés abiótico.

De las miles especies de leguminosas, el frijol (*Phaseolus vulgaris*) es la más importante para la alimentación humana, comprende el 50% de las leguminosas consumidas alrededor del mundo principalmente en Latinoamérica y África. En

algunos países como México y Brasil, el frijol es la principal fuente de proteínas en la dieta. (Broughton *et al.* 2003).

Algunos tipos de estrés abiótico como la salinidad, las deficiencias de nutrientes, los altos y bajos niveles de pH y toxicidades de metales son factores que limitan la producción de esta leguminosa. Muchos de los suelos de producción de frijol en África y Latinoamérica tienen un pH ácido lo que resulta en deficiencia de fósforo, toxicidad por alumninio (Al) y en algunos casos por manganeso (Mn). El aluminio es el metal más abundante en la corteza terrestre en donde se encuentra de forma no fitotóxica pero al solubilizarse en suelos ácidos, se convierte en uno de los factores más limitantes en la producción de cultivos (Abruna et al. 1982, Zheng 2010; Haynes et al. 2001).

1.3. El estrés de las plantas por la toxicidad por aluminio, por cobre y por otros metales

Los metales, tanto esenciales (cobre, fierro, manganeso y zinc) como no esenciales (cadmio, cobalto y mercurio) afectan el crecimiento y mantenimiento de las plantas. En general estos metales, al encontrarse en concentraciones superiores a las óptimas, inducen la acumulación de especies reactivas de oxígeno dando lugar a un daño de lípidos, proteínas y DNA (Schützendübel and Polle, 2002).

Uno de los síntomas principales en respuesta a metales como aluminio (Al), cobre (Cu), cadmio y mercurio es la inhibición del crecimiento de raíz, siendo el ápice de la radícula la zona más sensible de este efecto (Schutzendübel et al., 2001; Kochian et al., 2005; Rellán-Álvarez et al., 2006; Lequeux et al., 2010).

La expresión de diversos transportadores de metales es esencial para la tolerancia a la toxicidad por metales. Los transportadores de tipo ABC (ATP-binding casette) regula el transporte de Al y cadmio (Kim et al., 2007; Huang et al., 2012). La familia

NRAMP (natural resistance-associated macrophage protein) regula la respuesta a metales como Cd, Mn and Zn transport (DalCorso et al., 2010).

En este trabajo exploramos las respuestas del frijol a la toxicidad por aluminio y por cobre. A continuación se resume información sobre el efecto de la concentración elevada de estos metales en las plantas.

La toxicidad por aluminio ocasiona distintas respuestas en las plantas entre las cuales se encuentran:

-El síntoma primordial de las plantas al estar expuestas a una alta concentración de Al es la inhibición de la longitud de raíz, dando como resultado un sistema radical reducido y dañado lo cual lleva a una toma de nutrientes y minerales limitada. Esta respuesta es muy rápida (minutos) y es debida a que el Al inhibe la expansión y la elongación celular y a largo plazo la división celular también llega a verse afectada (Barceló *et al.* 2002; Matsumoto 2000).

- El sitio en donde se concentra el efecto de la toxicidad por Al es el ápice de la raíz, por lo que los mecanismos de tolerancia ocurren en esta región de la raíz (Ryan *et al.* 1993; Sivaguru *et al.* 1998).

-El aluminio afecta la dinámica del citoesqueleto, ya que interactúa con microtúbulos y filamentos de actina, lo cual podría ser una de las principales causas de la inhibición del crecimiento de raíz (Grabski *et al.* 1995; Sivaguru *et al.* 2003).

-Además del citoesqueleto hay otros sitios que son blanco del Al como la pared celular, membrana celular y el núcleo, debido la reactividad del Al (Kochian *et al.* 2005).

-La exposición a Al altera los niveles citosólicos de Ca2+, y pueden inhibir la enzima fosfolipasa C en la vía de los fosfoinosítidos asociado con la señalización de Calcio (Jones *et al*. 1995; 1998).

- El Al promueve la inducción de especies reactivas de oxígeno (ROS) dando lugar a un daño peroxidativo a membranas (Horst et al. 1992; Yamamoto et al 2001)

-La generación de ROS inducidas por Al se asocia con una disfunción de la mitocondria lo cual también se ha relacionado con la inhibición del crecimiento de la raíz (Yamamoto et al. 2002).

-El aluminio interfiere con el transporte y absorción de varios elementos esenciales como Cu, Zn, Ca, Mg, Mn, K, P y Fe. Además que la acidez del suelo promueve un antagonismo entre Ca y Al probablemente afectando la absorción de Ca por las plantas (Rout et al. 2001).

El mecanismo fisiológico de la plantas ante este estrés es la exudación de ácidos orgánicos por la raíz, éstos pueden ser oxalato, malato o citrato dependiendo de la especie de la planta.

Diversos estudios han mostrado que la liberación de ácidos orgánicos activada por Al es una respuesta muy rápida que se da en minutos, lo cual indica que ésta regulación y activación es a nivel de proteínas y no a un nivel de genes (Delhaize et al. 1993). Esta continua exudación de ácidos orgánicos incrementa su concentración en la superficie del ápice radicular a niveles suficientes para quelar y detoxificar una importante fracción del Al presente en la rizósfera que está en contacto con los ápices de la raíz previniendo su entrada a la misma; esta exudación continua así como la raíz va creciendo en el suelo, manteniendo su función de barrera quelante alrededor del ápice de raíz (Delhaize et al. 1993; Miyasaka et al. 1991).

Otro mecanismo fisiológico de resistencia a Al se basa en formar complejos y detoxificación del Al después de haber entrado a la planta. Estos complejos pueden ser formados con citrato u oxalato; estos complejos se forman en el citosol, el cual

está a un pH alrededor de 7, y lo protege de algún daño (Ma et al. 1997). La acumulación de Al interno generalmente se lleva a cabo en hojas (Ma et al., 2001) y es transportado desde el tallo por medio del xilema; para que se lleve a cabo este transporte los complejos generalmente son con citrato (Ma et al. 2000), lo cual implica un intercambio de ligando de oxalato a citrato. En algunos casos, para que el Al pueda ser transportado en el xilema y luego cambia de nuevo el ligando para formar complejos con oxalato cuando se acumula en hojas, ubicándose esta acumulación principalmente en vacuolas (Shen et al. 2002).

En cuanto a los mecanismos genéticos involucrados en la resistencia de Al se encuentran la expresión de transportadores de ácidos orgánicos como malato, siendo un ejemplo de este ALMT1 identificado en trigo (Sasaki et al. 2004). También genes involucrados con la capacidad de la planta para enfrentarse a las especies reactivas de oxígeno, como son genes codificantes para peroxidasas y superóxido dismutasas (Ezaki et al., 2000, Basu et al. 2001). También se ha reportado la inducción de genes codificantes para miembros de la familia de proteínas arabinogalactanos (AGP) las cuales son proteínas localizadas en pared celular relacionadas con el crecimiento y desarrollo de las plantas (Schultz et al. 2002).

Otro factor que afecta los cultivos, es el Cobre (Cu), el cual es un micronutriente, que llega a ser fitotóxico a concentraciones superiores a las óptimas, dando como resultado estrés oxidativo (Cuypers *et al.* 2002).Uno de los efectos descritos en las plantas cuando están en presencia de concentraciones altas de cobre, ocurre a nivel de las raíces, en relación a su crecimiento y morfología. El cobre tiende a acumularse en el tejido radical, con poco traslocamiento a la parte aérea (Marschner, 2011). Entre los principales síntomas se ha identificado un engrosamiento, oscurecimiento y agrietamiento de la cutícula de la raíz, daños en el meristemo así como una reducción de longitud de la raíz. La reducción en el número de pelos radicales también es otro de los síntomas característicos en

plantas expuestas a una alta concentración de Cu (Sheldon A. y Menzies NW; 2004).

A nivel celular, la quelación de Cu y la compartimentación del mismo dentro de la vacuola son estrategias que realiza la planta para proteger las funciones celulares bajo este estrés. La producción de componentes intracelulares como metalotioneínas y fitoquelatinas, incrementa conforme el Cu es acumulado en las raíces (Mocquot B. et al.; 1996).

El principal síntoma en las hojas en respuesta a la toxicidad por Cu es la clorosis la cual se relaciona con una disminución en la toma de hierro (Fe) ocasionada por el exceso de Cu (Lexmond and van der Vorm, 1981; Yau et al., 1991; Ouzounidou, 1995). En las hojas esta clorosis puede llegar a ocasionar necrosis y en el caso de raíces noduladas, el proceso de fijación de nitrógeno puede ser afectado disminuyendo el número de nódulos así como una disminución en la cantidad de el nitrógeno aprovechado en las asociaciones simbióticas con algunas especies de leguminosas (Broos et al. 2004; Stan et al., 2011; Tindwa et al. 2014).

En frijol se ha visto que el cobre induce un mecanismo de defensa antioxidativo en raíces y hojas primarias (Gupta et al. 1999, Cuypers et al. 2000).

En cuanto a la toxicidad por manganeso (Mn) los principales síntomas que se han observado en estudios en diferentes plantas son puntos color café necróticos en hojas, peciolos y tallos. Este síntoma comienza en las hojas más cercanas al tallo progresando a la parte aérea más alta. (Reichman S.M 2002). En cuanto aumenta el tiempo de exposición de las plantas al Mn, el moteo puede incrementar en cuanto a número y tamaño dando como resultado lesiones necróticas, oscurecimiento de hojas y muerte (Elamin and Wilcox, 1986a; Elamin and Wilcox, 1986b).

Otro síntoma común es conocido como “hojas arrugadas” lo cual ocurre en las hojas más jóvenes además de clorosis en los tallos y peciolos (Horst y Marschner, 1978; Wu, 1994; Bachman and Miller, 1995). En algunas especies la clorosis comienza en

las hojas más viejas progresando a las hojas jóvenes conforme pasa el tiempo de exposición (Gupta, 1972; Elamin and Wilcox, 1986a; Bachman and Miller, 1995); este síntoma comienza en los márgenes de las hojas continuando con las áreas intervenosas y si la toxicidad es aguda termina en la necrosis completa de las hojas (Bachman and Miller, 1995). En cuanto a las raíces expuestas a niveles tóxicos de Mn se ha observado una coloración café y algunas veces muestran agrietamientos (Le Bot et al., 1990; Foy et al., 1995).

Aunque el Cu y el Mn son metales que afectan principalmente a las raíces al estar en altas concentraciones en el suelo, la mayoría de los reportes acerca de los efectos de estos metales en las plantas se enfocan al daño en hojas y a los procesos fisiológicos que se llevan a cabo en las mismas. (Ouzounidou et al. 1995, Panou-Filotheou et al. 2001).

1.4 Los miRNAs: reguladores post-transcripcionales globales

La adaptación y/o tolerancia de las plantas a distintos tipos de estrés depende de una correcta regulación de la expresión génica, la cual puede ser a nivel transcripcional o post-transcripcional. Esta regulación puede ser a través de factores de transcripción (FT) y de RNAs pequeños y/o largos que no codifican proteínas (npcRNAs) (Jones –Rhoades et al. 2006; Hobert 2008; Ben Amor et al. 2009). Una de las clases de npcRNAs más estudiadas es la de los microRNAs (miRNAs). La biogénesis de miRNAs comprende un transcrito primario (primiRNA) de longitud variable (~ 1000 nt), el cual interacciona con la proteína DAWDLE (DDL) para ser transportado a un complejo proteico conformado por Dicer-like 1 (DCL1), HYL1 y Serrate, donde es procesado a un RNA más pequeño de ~ 200 nt (premiRNA), el cual posee una estructura de tallo y asa. El pre-miRNA es procesado por DCL1 para formar un RNA pequeño de doble cadena (miRNA/miRNA*), el cual es metilado en

su extremo 3' por la proteína HEN1 y exportado al citoplasma a través de la exportina HASTY. En el citoplasma la proteína AGO1 selecciona al miRNA maduro, que puede ser de 18-24 nt, mientras que el miRNA* es degradado por la exoribonucleasa SDN o en los cuerpos de procesamiento de RNAs (p-bodies). El miRNAs maduro es incorporado a la proteína AGO1, la cual forma parte del complejo de silenciamiento inducido por RNAs (RISC) donde los RNA mensajeros blancos complementarios de cada miRNAs son cortados o modificados para inhibir su traducción (Voinnet 2009). La mayoría de los miRNAs descritos en *Arabidopsis thaliana* se han vinculado con procesos de desarrollo de la planta, sin embargo, algunos reportes demuestran su participación en la adaptación de las plantas a distintos tipos de estrés abiótico como estrés oxidativo (miR398), a la deficiencia de fósforo (miR399), azufre (miR395), cobre (miR398), sequía (miR159 y miR169), así como al ataque de patógenos (miR393) (Jones –Rhoades et al. 2006; Phillips JR et al .2007; Fuji H et al. 2005; Sunkar et al. 2006; Reyes y Chua. 2007; Li WX et al. 2008, Covarrubias y Reyes 2009).

Aún se conoce poco sobre miRNAs de leguminosas y sus papeles regulatorios en distintos procesos fisiológicos. En *Medicago truncatula* (leguminosa modelo) se reportó que los miRNAs miR166 y miR169 están involucrados en el desarrollo de raíces laterales y nódulos (Combier et al. 2006; Boualem et al. 2008). Además en esta misma planta se han identificado algunos miRNAs específicos de órganos como raíz y nódulo (Lelandais-Briére et al., 2009). En raíces de soya, se han identificado 55 miRNAs de los cuáles 35 podrían ser únicos de soya (Subramanian et al. 2008). En frijol, se ha reportado la clonación de miRNAs específicos (Arenas-Huertero et al. 2009). Además en esta leguminosa se ha descrito el papel regulatorio de miR399 durante estrés por fósforo, demostrando que es un componente esencial en la vía de señalización del factor de transcripción PHR1 (Valdés-López et al. 2008). En el laboratorio se han obtenido resultados que indican que hay una expresión diferencial de miRNAs en diferentes órganos de plantas de frijol expuestas a distintos tipos de

estrés abiótico (deficiencia de P, N y Fe, toxicidad por Mn y pH ácido) (Valdés-López et al., 2010). Además se caracterizó el papel relevante de miR172 /AP2-1 de frijol en la simbiosis con rhizobia, demostrando el aumento en la expresión de este miRNA durante la etapa de infección por rhizobium misma que va aumentando durante el desarrollo del nódulo así como su importancia durante la fijación de nitrógeno. (Nova-Franco et al. 2015). Así mismo se realizó un análisis a nivel genómico basado en la secuencia genómica de frijol, de cinco bibliotecas de RNAs pequeños de distintos órganos de la planta y secuencias del degradoma que llevaron a identificar el catálogo de RNAs pequeños (miRNAs y phasiRNAs) y sus transcritos blancos y a proponer probables redes regulatorias miRNA-RNAm blanco, en nódulos fijadores de nitrógeno (Formey et al. 2015).

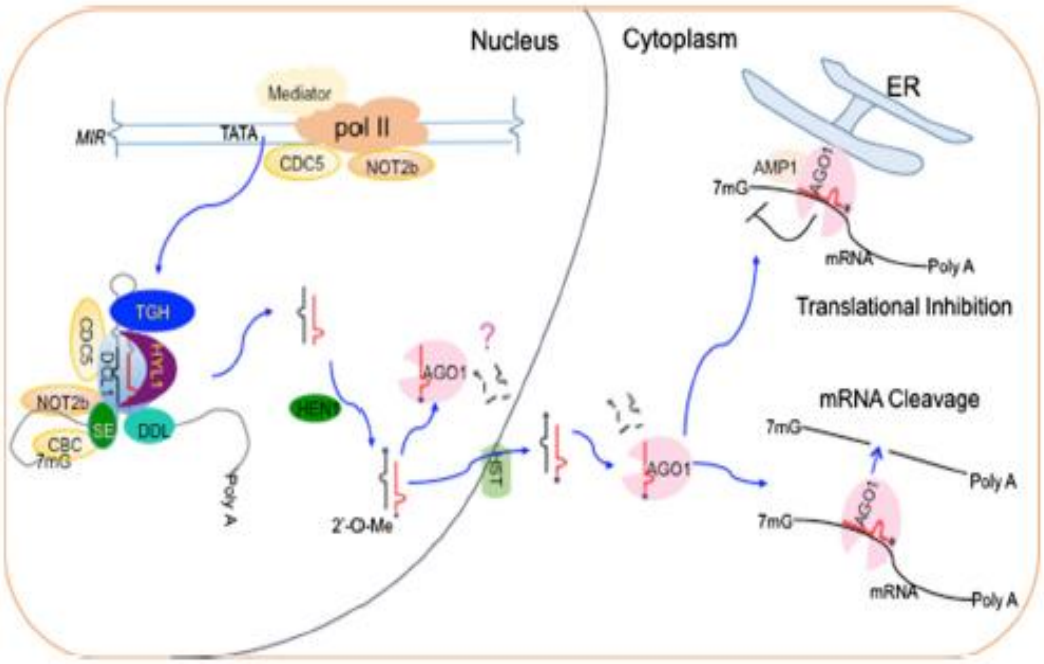

Figura 2. Biogénesis y acción de los miRNAs en plantas. (Xie et al. 2015)

1.5 Los miRNAs como reguladores de la respuesta a toxicidad por metales

La toxicidad por metales es un estrés importante que afecta la producción de cultivos. Este incluye a los metales que son esenciales para las plantas (cobre, hierro, zinc, manganeso) y metales no esenciales (cadmio, aluminio, cobalto, mercurio). Un efecto primario común ante altas concentraciones de metales como aluminio, cobre, cadmio o mercurio es la inhibición del crecimiento de las raíces. La toxicidad por metales desencadena la acumulación de especies reactivas de oxígeno que daña los lípidos, las proteínas y el ADN.

La respuesta de las plantas a la toxicidad por metales involucra varios procesos biológicos que requieren una regulación fina y precisa a nivel transcripcional y posttranscripcional.

Los microRNAs (miRNAs) son RNAs no codificantes de 21 nucleótidos que regulan la expresión génica a nivel post-transcripcional. El miRNA, incorporado a un complejo de silenciamiento inducido por RNA, promueve el corte de su RNAm blanco, que es reconocido por una complementariedad de bases casi perfecta. En las plantas, la regulación de miRNA está implicada en el desarrollo y también en respuestas de estrés biótico y abiótico. La mayoría de los RNAm blanco para los miRNAs responsivos a metales en plantas, son factores de transcripción.

La información sobre los miRNAs sensibles a los metales en diferentes plantas indica posibles papeles reguladores importantes de miR319, miR390, miR393 y miR398. El blanco de miR319 es el factor de transcripción TCP, implicado en el control del crecimiento. MiR390 ejerce su acción a través de la biogénesis de los RNA pequeños de interferencia “trans-acting” que, a su vez, regulan los factores de respuesta a las auxinas. MiR393 actúa sobre los receptores de auxina TIR1 / AFBs y un factor de transcripción de bHLH.

El miR398 tiene un papel crucial en el control del estrés oxidativo generado después de una alta exposición de cobre o hierro, debido a su acción sobre las superóxido dismutasas Cu / Zn.

En el siguiente artículo de revisión profundizamos sobre el papel de los principales miRNAs identificados como responsivos a metales, así como su especificidad por órgano y el potencial como reguladores esenciales en las respuestas generales a toxicidad por metales.

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MicroRNAs as regulators in plant metal toxicity response

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Metal toxicity is a major stress affecting crop production. This includes metals that are essential for plants (copper, iron, zinc, manganese), and non-essential metals (cadmium, aluminum, cobalt, mercury). A primary common effect of high concentrations of metal such as aluminum, copper, cadmium, or mercury is root growth inhibition. Metal toxicity triggers the accumulation of reactive oxygen species leading to damage of lipids, proteins, and DNA. The plants response to metal toxicity involves several biological processes that require fine and precise regulation at transcriptional and post-transcriptional levels. MicroRNAs (miRNAs) are 21 nucleotide non-coding RNAs that regulate gene expression at the post-transcriptional level. A miRNA, incorporated into a RNA-induced silencing complex, promotes cleavage of its target mRNA that is recognized by an almost perfect base complementarity. In plants, miRNA regulation is involved in development and also in biotic and abiotic stress responses. We review novel advances in identifying miRNAs related to metal toxicity responses and their potential role according to their targets. Most of the targets for plant metal-responsive miRNAs are transcription factors. Information about metal-responsive miRNAs in different plants points to important regulatory roles of miR319, miR390, miR393, and miR398. The target of miR319 is the TCP transcription factor, implicated in growth control. miR390 exerts its action through the biogenesis of trans-acting small interference RNAs that, in turn, regulate auxin responsive factors. miR393 targets the auxin receptors TIR1/AFBs and a bHLH transcription factor. Increasing evidence points to the crucial role of miR398 and its targets Cu/Zn superoxide dismutases in the control of the oxidative stress generated after high copper or iron exposure.

Keywords: microRNAs, metal toxicity, abiotic stress

PLANT RESPONSE TO METAL TOXICITY

Plants are constantly exposed to numerous abiotic and biotic stresses. One important abiotic stress is metal toxicity. Heavy metals such as copper (Cu), iron (Fe), and zinc (Zn) are essential for physiological and biochemical processes, and metals such as cadmium (Cd), cobalt (Co), mercury (Hg), and aluminum (Al) are non-essential. Nevertheless, high concentrations of any metal type is toxic for the plant.

One of the primary symptoms of toxicity of metals such as Al, Cu, Cd, and Hg is root growth inhibition, with the root apex being the most sensitive part of the root (Schützendübel et al., 2001; Kochian et al., 2005; Rellán-Álvarez et al., 2006; Lequeux et al., 2010).

Plant responses to cope with metal toxicity include the synthesis of different proteins involved in detoxification, such as phytochelatins and metallothioneins (Cobbett and Goldsbrough, 2002). Root exudation of organic acids – citric, oxalic, malic – and amino acids – histidine – to the rhizosphere is an important physiological response since these compounds can form complexes with the heavy metals leading to detoxification (Hall, 2002).

The expression of several metal transporters is essential for tolerance to metal toxicity. The ABC-transporters (ATP-binding cassette) family mediates the transport of Al and Cd (Kim et al., 2007; Huang et al., 2012). The NRAMP (natural resistance-associated macrophage protein) family regulates response to Cd, manganese (Mn), and Zn transport (DalCorso et al., 2010). The CDF (cation diffusion facilitator) family, involved in cytoplasmic efflux and vacuolar sequestration of divalent metal cations, plays a role in Zn, Cd, Co, nickel (Ni), or Mn metal toxicities (Krämer et al., 2007). Transporters from the P-type ATPases ion pumps systems have been linked to the transport of heavy metals such as Cu, Zn, Cd, and lead (Pb; Axelsen and Palmgren, 2001; Andres-Colas et al., 2006; Lee et al., 2007).

Metal toxicity stress triggers the accumulation of ROS (reactive oxygen species), unbalancing the activity of antioxidative enzymes that are up-regulated by this stress (Romero-Puertas et al., 2007). Oxidative stress leads to damage of lipids, proteins, and DNA (Schützendübel and Polle, 2002).

Plant response to abiotic stress such as metal toxicity involves a precise regulation of gene expression at the transcriptional and post-transcriptional levels. Regulation can be achieved by transcription factors (TF) from different families such as myeloblastosis protein (MYB), basic leucine Zipper (bZIP), ethylene-responsive factor (ERF), and WRKY (Jacoby et al., 2002; Krämer et al., 2007; Wei et al., 2008; Farinati et al., 2010). Cis-acting elements have been identified in the promoter regions of metal-responsive genes such as *parA*, an auxin-regulated gene, involved in Cd-response in *Nicotiana tabacum* (Kusaba et al., 1996). The Cu-response element (CuRE) with a consensus GTAC was identified in the promoter of the coprogen oxidase and the

cytochrome c_6 genes from the green algae *Chlamydomonas reinhardtii* (Quinn et al., 2000). Two promoter regions of the PvSR2 gene from *Phaseolus vulgaris* contain heavy metal-responsive elements (HMREs; Qi et al., 2007).

Small and/or large non-protein coding RNAs (npcRNAs) may be involved in the regulation/signaling of metal toxicity response (Jones-Rhoades et al., 2006; Hobert, 2008; Ben Amor et al., 2009). One of the most studied classes of npcRNAs is the micro RNAs (miRNAs). miRNAs are 21 nucleotide npcRNAs that regulate gene expression at the post-transcriptional level in plants. A precursor miRNA (pre-miRNA) with imperfect hairpin structure is processed into a mature miRNA and this is incorporated into a RNA-induced silencing complex (RISC) that promotes degradation/cleavage of the corresponding target mRNA(s), recognized by an almost perfect base complementarity with the miRNA (Jones-Rhoades et al., 2006). Though most of the miRNAs identified in *Arabidopsis thaliana* are related to plant development, there is evidence of the role of miRNAs in the plant response to different abiotic stresses including metal toxicity (Jones-Rhoades and Bartel, 2004; Fujii et al., 2005; Sunkar et al., 2006; Phillips et al., 2007; Reyes and Chua, 2007; Li et al., 2008).

miRNAs AND THEIR TARGETS IN METAL TOXICITY

Analyses of small RNAs expression profiles performed in plants exposed to metal toxicities has shown the differential expression of miRNA and their targets, thus indicating their possible role in regulation/signaling pathways. To determine the role of a specific miRNA it is important to analyze the function of its target(s) and their possible interactions with signaling pathways related to metal toxicity responses. Most of the targets predicted for metal-responsive conserved miRNAs are TF mainly involved in plant development (**Table 1**). Up-regulation of a certain miRNA resulting in its target degradation might indicate the target role as a negative regulator of metal toxicity response. Recent high-throughput genomic technologies as well as other genetic-genomic approaches have increased our current knowledge of miRNAs and their target in signaling pathways for metal toxicities response in several plant species (Sunkar and Zhu, 2004; Phillips et al., 2007; Huang et al., 2009; Ding et al., 2011; Chen et al., 2012; Zhou et al., 2012).

In regard to miRNAs that respond to Cd-toxicity the conserved miRNAs: miR160, miR164, and miR167 and the novel of Osa-miR602 and Osa-miR604 were identified in a library of small RNAs from rice seedlings exposed to Cd (Huang et al., 2009). Osa-miR602 is up-regulated in rice roots exposed for 12 h to high Cd; its predicted target is a xyloglucan endotransglocosylase/hydrolase. Osa-miR604, which was up-regulated in leaves treated with toxic levels of Cd for 6 h, down-regulated a lipid transfer protein (LPT; Huang et al., 2009). This type of protein is responsive to environmental stresses and to abscisic acid, salicylic acid, ethylene, and methyl jasmonate that has been proposed to participate in cutin and wax assembly and in defense of plant against pathogens (Arondel et al., 2000; Kim et al., 2008). Rice microarray data showed that miR528 is up-regulated, while miR162, miR166, mir171, miR390, miR168, and miR156 families were down-regulated under Cd stress (Ding et al., 2011). The search of possible metal-responsive cis-acting elements revealed that a MRE-like sequence (5′-TGCGCNC-3′) is present in promoter regions of most of the Cd-responsive miRNA genes (Ding et al., 2011). Other cis-acting elements related to different abiotic stresses such as ARE (anaerobic-responsive element); ABRE (ABA-responsive element); GARE (gibberellins-responsive element); ERE (ethylene-responsive element); HSE (heat stress-responsive element); and LTR (low temperature-responsive element) were also identified in these miRNA genes promoters, thus implying that these miRNAs could be responsive to other stress signals besides metal toxicity (Ding et al., 2011). In roots of *Brassica napus* miR393, miR171, miR156, and miR396 are down-regulated after Cd exposure (8 h; Xie et al., 2007).

Table 1 | Metal toxicity-responsive miRNAs.

Related metal toxicity	miRNA	Targets	Reference
Cd, Hg, Al, Mn	miR319	TCP transcription factors	Zhou et al. (2008), Valdés-López et al. (2010), Chen et al. (2012)
Cd, Hg, Al	miR171	SCL transcription factors	Xie et al. (2007), Zhou et al. (2008, 2012)
Cd, Hg, Al	miR390	TAS3	Ding et al. (2011), Chen et al. (2012), Zhou et al. (2012)
Cd, Hg, Al	miR393	TIR1/AFBs (F-box auxin receptors) and bHLH transcription factors	Xie et al. (2007), Zhou et al. (2008)
Cd, Hg, Al	miR396	GRF transcription factors	Xie et al. (2007), Chen et al. (2012), Zhou et al. (2012)
Cd, Hg, Mn	miR167	Auxin responsive factors (ARFs)	Huang et al. (2009), Valdés-López et al. (2010), Zhou et al. (2012)
Cd, Hg	miR164	NAC, CUP trancription factors	Huang et al. (2009), Zhou et al. (2012)
Cd, Al	miR160	Auxin responsive factors (ARFs)	Huang et al. (2009), Chen et al. (2012)
Cd	miR156	SBP transcription factors	Xie et al. (2007), Ding et al. (2011)
Cu, Fe, Mn	miR398	CSD, COX5b.1, CCS	Sunkar et al. (2006), Zhou et al. (2008), Valdés-López et al. (2010)
Hg, Mn	miR172	AP2 transcription factors	Valdés-López et al. (2010), Zhou et al. (2012)
Mn	miR397	Laccases	Valdés-López et al. (2010)

In leaves of the model legume *Medicago truncatula* miR393, miR171, miR319, and miR529 are up-regulated, while miR166 and miR398 are down-regulated after Cd, Hg, and Al exposure (24 h; Zhou et al., 2008). A high-throughput small RNA-sequencing approach revealed that miR159, miR160, miR319, miR396, and miR390 were down-regulated in response to Al (Chen et al., 2012). More recently a study using a similar approach identified Hg-toxicity responsive miRNAs such as the miR167, miR172, miR169, miR164, miR395 families that are up-regulated, whereas the miR396, miR390, and miR171 are down-regulated in this legume. In addition, new *M. truncatula* Hg-responsive miRNAs were identified such as miR2681 targets the transcripts coding TIR-NBS-LRR disease resistance proteins (Zhou et al., 2012).

Our group has reported the miRNA expression profile in common bean (*P. vulgaris*), the most important legume for human consumption. Using a miRNA-macroarray hybridization approach we identified miRNAs that respond to nutrient deficiencies and to Mn-toxicity in different plant organs. In common bean plants exposed to high Mn miR397 is down-regulated in leaves, miR319 and miR398 are up-regulated in roots and nodules, miR172 is up-regulated in nodules and miR167 is up-regulated in roots (Valdés-López et al., 2010). Recently, the identification and characterization of miRNAs in *P. vulgaris* by high-throughput sequencing has been completed (Peláez et al., 2012).

Current information about metal-responsive miRNAs in different plants indicates the common relevant role of miR319, miR390, miR393, and miR398.

ROLES OF miR319, miR390, miR393, AND miR398

miR319

Plant growth and senescence are processes affected by metal toxicity (Maksymiec, 2007). Common responses of shoots to Al- and Cu-toxicity include cellular and ultrastructural changes in leaves, decreased photosynthetic activity leading to chlorosis and necrosis of leaves, total decrease in leaf number and size, and decreased shoot biomass (Thornton et al., 1986; Lanaras et al., 1993; Maksymiec, 1997; Panou-Filotheou et al., 2001). In addition, Cu-toxicity leads to rapid senescence in leaves (Luna et al., 1994). Interestingly, miR319 and its target TCP (Teosinte Branched/Cycloidea/PCF) TF (**Table 1**), implicated in growth control, have shown differential expression in most of the studies of miRNAs responding to metal toxicity. Members of the TCP family bind to promoter elements which are essential for the expression of the proliferating cell nuclear antigen (PCNA) gene (Kosugi and Ohashi, 1997). Other TCPs are involved in the morphogenesis of shoot lateral organs (Li et al., 2005). Lately, it has been demonstrated that miR319 plays a role on leaf senescence through the regulation of TCPs that positively control leaf senescence via JA biosynthesis and important senescence positive regulators like WRKY53 (Schommer et al., 2008).

Figure 1 depicts the mode of action of miR319 and TCP. In leaves, high Cd, Hg, and Al induce miR319 leading to the degradation of TCP thus affecting growth and senescence. In the roots, this miRNA is repressed in response to Al while it is induced in Mn-toxicity (Valdés-López et al., 2010; Chen et al., 2012). The opposite regulation of miR319 could be due to the

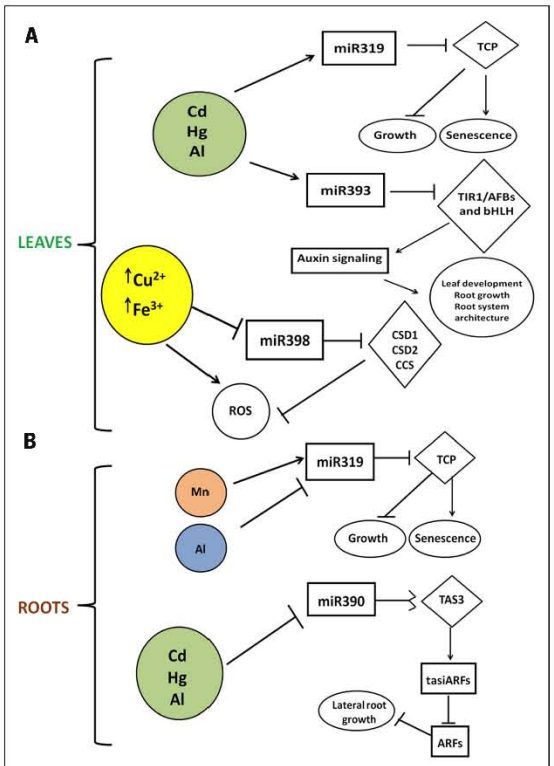

FIGURE 1 | Schematic representation of the mode of action of miR319, miR390, miR393, and miR398 in response to metal toxicity. **(A)** Regulation in leaves. **(B)** Regulation in roots. miRNAs are in rectangles while their targets are in diamonds. Arrows indicate up-regulation and blunted lines indicate down-regulation.

different plant species and/or the different time of exposure and metal concentration used. When both metals are abundant in the ground Al may exert an antagonistic effect on the uptake of Mn thus ameliorating Mn-toxicity (Blair and Taylor, 1997; Yang et al., 2009). There are no reports about the regulation of miR319 when plant roots are exposed to the combination of Al and Mn; we find difficult to speculate about this issue since specific effects in the plant would depend on several variables (concentration, time of exposure, environmental conditions).

miR390

miR390 and its target TAS3 (**Table 1**) are related to metal toxicity response in different plants. The miR390-induced cleavage of TAS3 transcript initiates ta-siRNAs (trans-acting small interference RNAs) biogenesis, leading to the degradation of ARFs (auxin response factors) that play critical roles in lateral root development (Marin et al., 2010). miR390 is repressed in roots of plants under Cd, Al, and Hg toxicities, which would lead the accumulation of intact TAS3 transcript and the decrease of tasiARFs resulting in

the inhibition of lateral root growth (Chen et al., 2011; Zhou et al., 2012; **Figure 1**).

Arabidopsis plants exposed to high Cu show decreased primary root growth and increased short lateral root density. Also, changes in auxin and cytokinin accumulation and in mitotic activity within the primary and secondary root tips were observed in Cu-exposed plants (Lequeux et al., 2010). We may speculate that miR390 and its targets could also respond to Cu-toxicity, but no information about miR390 regulation in this stress is available.

miR393

miR393 is regulated by Cd, Hg, and Al toxicities (Xie et al., 2007; Zhou et al., 2008). These metals induce miR393 in leaves, which would lead to the repression of its targets the F-box auxin receptors TIR1/AFBs and bHLH transcription factors (**Table 1;** Jones-Rhoades and Bartel, 2004; Navarro et al., 2006). TIR1 positively regulates auxin signaling, its level would be low when the miR393 increases leading to an inhibition of auxin signaling. Studies have shown the importance of miR393 regulation of leaf development, root system architecture, and root growth (Vidal et al., 2010; Si-Ammour et al., 2011; Chen et al., 2011; **Figure 1**). This miRNA also responds to bacterial infection with *Pseudomonas syringae* and to salinity (Navarro et al., 2006; Gao et al., 2011).

The root is the main organ affected by high concentration of metals such as Cu, Cd, Cu, Hg, and Al in the soil, and the common phenotypic response is changes in architecture (Karataglis et al., 1988; Kahle, 1993; Kochian et al., 2005). In such responses miR393 and miR390 may play relevant regulatory roles.

miR398

miR398 was the first miRNA identified to be regulated by oxidative stress. Its targets are the Cu/Zn superoxide dismutases (CSD) enzymes: cytosolic CSD1 and plastidic CSD2 such as the COX5b.1, the 5b subnunit of mitochondrial cytochrome oxidase (**Table 1**). CSDs are enzymes that scavenge superoxide radicals to release molecular oxygen and hydrogen peroxide, which are less ROS (Kliebenstein et al., 1998). The CSD2 mRNA sequence that is complementary to miR398 is within the coding sequence while CSD1 and COX5b.1 transcripts contain miR398 complementary sequence in the 5′UTR (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004).

The miR398 promoter contains the GTAC sequence that has an important feature in Cu responsiveness (Yamasaki et al., 2009). This motif is recognized by the SPL7 TF that binds to the promoter and regulates the expression of miR398. In addition, this TF regulates the expression of other Cu-related miRNAs: miR397, mir408, and miR857 (Ding and Zhu, 2009). The GTAC promoter sequence is also present in other important Cu-responsive genes such as *CPX1* (coproporphyrinogen oxidase) and Cyc6 (cytochrome 1 c6) that improve the ability to assimilate Cu under Cu deficiency (Hill and Merchant, 1995; Quinn et al., 2000).

Oxidative stress suppresses miR398 expression that is essential for the accumulation of CSD1 and CSD2 transcripts (**Figure 1**). miR398 is down-regulated upon exposure to heavy metals such as Cu^{2+} and Fe^{3+} (Sunkar et al., 2006) that are involved in Fenton-type reactions and have potential to generate hydroxyl radicals (Dietz et al., 1999; Estevez et al., 2001). In addition, miR398 is down-regulated in response to high light and MV (methyl viologen) when the levels of CSD1 and CSD2 transcripts usually are increased (Sunkar et al., 2006; Yamasaki et al., 2007).

The co-supressed transgenic lines, over-expressing miR398 precursors, display an increased tolerance to Cu^{2+} and to MV stresses in terms of seedling development and lipid peroxidation rates (Sunkar et al., 2006). In contrast, some reports show that over-expression of miR398b and miR398c is possible and the over-expressing lines show reduced CSD1 and CSD2, but not COX5b.1, mRNA, and protein levels (Yamasaki et al., 2007; Dugas and Bartel, 2008).

Sucrose is an important signal for miR398 expression; it positively regulates this miRNA accumulation. In agreement CSD1 and CSD2 protein levels, but not COX5b.1, levels decreased as sucrose is increased. The increase in the miR398 level by sucrose is maintained both in the presence and in the absence of Cu, thus miR398 regulation by sucrose and by Cu are independent (Dugas and Bartel, 2008).

The Cu chaperone for superoxide dismutase (CCS1) was recently identified as a miR398 target; its mRNA cleavage is mediated by miR398 when Cu is scarce (Beauclair et al., 2010). CCS1 delivers Cu to CSDs (Abdel-Ghany et al., 2005). Beauclair et al. (2010) proposed that the regulation of CCS1 by miR398 could be responsible for the unchanged protein levels of CSD1 and CSD2 in studies of plants expressing miR398-resistant forms of CSD1 or CSD2 (Dugas and Bartel, 2008).

Furthermore, miR398 has a role in biotic stress. This miRNA decreases in *Arabidopsis* leaves infiltrated with avirulent strains of *Pseudomonas syringae* pv tomato, CSD1 was negatively correlated with miR398 levels. Avirulent strains induce a biphasic accumulation of ROS (oxidative burst) leading to the accumulation of ROS at the beginning of the hypersensitive response and at a second phase accompanied by local cell death (Lamb and Dixon, 1997; Wojtaszek, 1997; Torres et al., 2006). Plants exposed to virulent strains do not show drastic changes in the levels of miR398, which could be due to the absence of the oxidative burst or to the presence of just the initial accumulation of ROS (Jagadeeswaran et al., 2009) and the negative regulation of the signaling cascade induced by the T3SS proteins that target key cellular functions (Jones and Dangl, 2006).

Apparently, the common signal of the abiotic stresses such as Cu^{2+}, Fe^{3+}, high light, MV, ozone, salinity, and the biotic stress (avirulent strains) is the accumulation of ROS. The generation of ROS is one of the common responses to metal toxicities as well as the synthesis of active antioxidative enzymes. Both responses vary among different metal exposures (Sharma and Dietz, 2009), the specific response of miR398 or other ROS-responsive miRNAs may vary according to the metal and to the time of exposure to the stress (**Figure 1**).

CONCLUDING REMARKS

The identification and analysis of miRNAs responsive to different metal toxicities has provided information about their possible relations in the networks involved in plant adaptation to these abiotic stresses. These studies are recent so we can predict the discovery of additional novel metal stress-responsive miRNAs.

Further research is needed to deeply understand the role of miRNAs and their targets, mainly TFs, as main players in signaling pathways of plant responses to environmental changes. This should take into account that plant species varying in growth habits and genotypic backgrounds may have differential responses of miRNAs to metal stresses. More detailed analysis on the kinetics of miRNAs and target regulation over a time-course of metal exposure, including different metal concentration and combinations of metals, will be helpful for obtaining better mechanistic insights into the roles of miRNAs in stress-regulatory networks. To elucidate novel roles of miRNAs in the response to metal toxicities it is important to perform phenotypic analysis of plants with modulated expression of a specific miRNA and/or its respective targets. A better understanding of the role of miRNAs during metal stress will contribute to the better design of strategies aimed at improving stress tolerance of crop plants.

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2. OBJETIVO GENERAL

Analizar las respuestas a la toxicidad por metales en plantas de frijol (*Phaseolus vulgaris*), a nivel fenotípico y de expresión de miRNAs proponiendo su posible participación en vías de regulación en el desarrollo de raíces y/o nódulos bajo este estrés.

2.1 OBJETIVOS PARTICULARES

- Caracterización de las respuestas fisiológicas de las raíces y nódulos de plantas de frijol expuestas a estrés por toxicidad por aluminio.
- Evaluación de la expresión diferencial de miRNAs en nódulos y raíces de plantas de frijol crecidas en estrés de toxicidad por aluminio, durante distintos periodos de tiempo.
- Caracterización de las respuestas fisiológicas de las raíces y nódulos de plantas de frijol expuestas a estrés de toxicidad por cobre.
- Evaluación de la expresión diferencial de miRNAs en nódulos y raíces de plantas de frijol crecidas en estrés de toxicidad por Cobre, durante distintos periodos de tiempo.
- Análisis de los miRNAs de frijol: miR398 y pvu-miR1511 como reguladores de las respuestas a la toxicidad por metales.

3. RESULTADOS

3.1 Toxicidad por Aluminio en plantas de frijol en simbiosis con rhizobia: fenotipo y perfil de expresión de miRNAS.

La toxicidad por aluminio (Al) es muy común en suelos ácidos donde se produce el frijol (*Phaseolus vulgaris*), la leguminosa más importante para el consumo humano, y es un factor limitante para la producción de cultivos y la fijación simbiótica de

nitrógeno. En este trabajo se caracterizaron las respuestas de los nódulos de plantas de frijol inoculadas con *Rhizobioum tropici* CIAT899 y las respuestas en las raíces de plantas fertilizadas con nitrato, expuestas a exceso de Al en pH bajo, durante períodos largos o cortos.

Se observó una reducción del 43-50% en la actividad de nitrogenasa, lo cual indica que la toxicidad por Al (Alt) afectó considerablemente la fijación de nitrógeno en el frijol.

Las raíces y nódulos de frijol mostraron síntomas característicos de Alt. En los nódulos maduros se observó acumulación de Al y lipoperoxidación en la zona infectada, mientras que la deposición de callosa y la muerte celular ocurrieron principalmente en el córtex del nódulo.

Los mecanismos reguladores de las respuestas de las plantas a la toxicidad por metales implican la participación de los microRNAs (miRNAs) as, entre otros reguladores. Se utilizó un enfoque de hibridación de macroarreglos de miRNAs. Este an´lisis resultó en la identificación de 28 miRNAs que responden a Al en los nódulos (14 de ellos se inducen).

Se validó la expresión de ocho miRNAs responsivos a Al mediante qRT-PCR (PCR cuantitativa de la transcriptasa inversa) en raíces y nódulos expuestos a una alta concentración de Al durante periodos cortos y largos. La correlación inversa de expresión entre el miRNA y el RNAm blanco (estrés: control) se observó en todos los casos. En general, los miRNAs mostraron una respuesta más temprana en las raíces que en los nódulos. Algunos de los miRNA identificados como responsivos a Alt en frijol, también han mostrado una expresión diferencial en otras especies de plantas sometidas a condiciones similares de estrés.

Los nodos miRNA / RNAm analizados en este trabajo están involucrados en vías de señalización relevantes, por lo que proponemos que la participación de miR164

/ NAC1 (NAM / ATAF / CUC transcription factor) y miR393 / TIR1 (TRANSPORT INHIBITOR RESPONSE 1 like protein) que participan en la regulación de auxinas; y de miR170 / SCL (SCARECROW-like protein transcription factor) que participan en la señalización de giberelinas, son relevantes para la respuestas y adaptación del frijol a estrés por Al. En el siguiente artículo describimos los datos que proporcionan una base para evaluar los papeles individuales de miRNAs en la respuesta de los nódulos de frijol común a Alt.

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Responses of symbiotic nitrogen-fixing common bean to aluminum toxicity and delineation of nodule responsive microRNAs

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Aluminum (Al) toxicity is widespread in acidic soils where the common bean (*Phaseolus vulgaris*), the most important legume for human consumption, is produced and it is a limiting factor for crop production and symbiotic nitrogen fixation. We characterized the nodule responses of common bean plants inoculated with *Rhizobioum tropici* CIAT899 and the root responses of nitrate-fertilized plants exposed to excess Al in low pH, for long or short periods. A 43–50% reduction in nitrogenase activity indicates that Al toxicity (Alt) highly affected nitrogen fixation in common bean. Bean roots and nodules showed characteristic symptoms for Alt. In mature nodules Al accumulation and lipoperoxidation were observed in the infected zone, while callose deposition and cell death occurred mainly in the nodule cortex. Regulatory mechanisms of plant responses to metal toxicity involve microRNAs (miRNAs) along other regulators. Using a miRNA-macroarray hybridization approach we identified 28 (14 up-regulated) Alt nodule-responsive miRNAs. We validated (quantitative reverse transcriptase-PCR) the expression of eight nodule responsive miRNAs in roots and in nodules exposed to high Al for long or short periods. The inverse correlation between the target and miRNA expression ratio (stress:control) was observed in every case. Generally, miRNAs showed a higher earlier response in roots than in nodules. Some of the common bean Alt-responsive miRNAs identified has also been reported as differentially expressed in other plant species subjected to similar stress condition. miRNA/target nodes analyzed in this work are known to be involved in relevant signaling pathways, thus we propose that the participation of miR164/NAC1 (NAM/ATAF/CUC transcription factor) and miR393/TIR1 (TRANSPORT INHIBITOR RESPONSE 1-like protein) in auxin and of miR170/SCL (SCARECROW-like protein transcription factor) in gibberellin signaling is relevant for common bean response/adaptation to Al stress. Our data provide a foundation for evaluating the individual roles of miRNAs in the response of common bean nodules to Alt.

Keywords: common bean, aluminum toxicity, symbiotic nitrogen fixation, legume-rhizobia, nodules, microRNAs

Introduction

Legumes are second only to the Graminiae in their importance to humans. Grain legumes provide more than one–third of humankind nutritional nitrogen requirements. A hallmark trait of legumes is their ability to establish mutualistic symbioses with nitrogen-fixing bacteria collectively known as rhizobia. Symbiotic nitrogen fixation (SNF) by differentiated bacteroids takes place in specialized rhizobia-induced root nodules. This process involves a tight association between the two symbionts. SNF reduces the cost of legume cultivation and makes them valuable source of soil nitrogen to other crops (Graham and Vance, 2003).

Common bean (*Phaseolus vulgaris*) is the most important legume for human consumption. In Mexico and other countries common bean are staple crops serving as the primary source of protein in the diet. Common bean is mainly grown by small landholders in tropical areas of Latin America and Africa; soil acidity in the tropics is a major constraint for crop productivity (Broughton et al., 2003; Graham et al., 2003). It has been estimated that almost 50% of the world's potentially arable lands are acidic and the American continent accounts for 40% of the world's acid soils (von Uexküll and Mutert, 1995). Poor crop growth in acid soils is due usually to a combination of metal toxicity and nutrient deficiency, primarily toxic levels of aluminum (Al) and manganese (Mn) and suboptimal levels of phosphorus (P) (von Uexküll and Mutert, 1995; Kochian et al., 2004). Research from our group, based on transcriptomics, metabolomics, and miRNA profiles, has contributed to define the response of common bean to P deficiency and to Mn toxicity (Hernández et al., 2007, 2009; Valdés-López et al., 2008, 2010; Ramìrez et al., 2013). In this work we analyze the response of SNF common bean to Al toxicity (Alt), an important constraint for common bean crop production in Mexican acidic soils.

Al toxicity is the primary growth-limiting factor in acidic soils. Solubility of Al is pH-dependent, at soil pH values below 5.5 Al^{3+} is solubilized into soil solution and this is the most important rhizotoxic Al species. High levels of Al^{3+} in the soil inhibit root growth and function, increase the risk of plants to succumb to drought and to mineral deficiencies and reduce crop production (Kochian et al., 2004). Considerable advances have been made to understand the mechanisms of Alt, but some aspects remain unclear (reviewed by Kochian et al., 2005; Vitorello et al., 2005). Some Alt symptoms and responses are detectable shortly (seconds or minutes) after Al exposure, while others are only discernable after long-term (hours or days) exposure. The timing and type of plant responses to Alt shows high variability among plants species/genotypes, different experimental conditions used for analysis or to diverse natural/environmental conditions for crop production (Kochian et al., 2005). The primary and earliest symptom of Alt is a rapid inhibition of root growth and lateral root formation; Al disrupts root cell expansion and elongation leading to inhibition of cell division (Frantzios et al., 2001). A recent detailed analysis by Kopittke et al. (2015) identified that the primary lesion of Al is apoplastic, reducing root growth at very short period by binding to the walls of outer cells and directly inhibiting their loosening in the elongation zone. The root apex is considered to be the primary target of Al stress, where Al affects diverse cellular processes and signal-transduction pathways due to its high reactivity. Al has strong affinity to negatively charged plasma membrane, thus can modify membrane structure and cause depolarization. In addition, Al ions can displace other cations that may form bridges between the phospholipid head groups of the membrane bilayer altering membrane fluidity and homeostasis, leading to disturbance of ion-transport processes (Akeson et al., 1989; Lindberg et al., 1991). Al induces accumulation of reactive oxygen species (ROS) that cause peroxidative damage to lipids and lead to mitochondrial dysfunction that could be related to root growth inhibition (Yamamoto et al., 2001). Oxidative damage leads to a disturbance of cellular homeostasis that may result in cell death; Alt induced cell death has been observed in the elongation zone of roots from metal-stressed plants (Yamamoto et al., 2003). After analyzing the response to Alt of the legume *Lotus corniculatus*, Navascués et al. (2011) concluded that oxidative stress is a consequence not a cause of Alt. The apoplast accumulation of the polysaccharide callose has also been documented as a characteristic symptom of Alt in plants, it may lead to cellular damage by inhibiting intercellular transport through plasmodesmatal connections and to cell wall rigidification that promotes growth inhibition of the root (Horst et al., 1997; Sivaguru et al., 2000). Clear evidence from different plant genotypes indicates that a mechanism for Al tolerance is Al-exclusion based on carboxylates exudation form roots apex; Al-carboxylate complexes are not transported into roots or across membranes (Kochian et al., 2005; Vitorello et al., 2005).

Acidity and high Al in tropic and temperate soils pose an additional challenge for legumes because their symbiotic rhizobia are sensitive to acidity. Reduced growth in acidic/Alt condition has been observed in different rhizobial species, both in laboratory conditions and in natural environments (Graham et al., 1994; O'Hara and Glen, 1994; Paudyal et al., 2007; Avelar Ferreira et al., 2012). Root nodule bacteria can be more sensitive to low pH and Alt than their legume host, and so their survival and persistence in acidic soils results in diminished infection, nodulation and SNF. Total or partial nodulation inhibition in legumes exposed to high Al has been reported for several species such as common bean (*P. vulgaris*), clover (*Trifolium repens*), *Stylosanthes* species and other tropical legumes (De Carvalho et al., 1981; Franco and Munns, 1982a; Wood et al., 1984; Paudyal et al., 2007).

Regulatory mechanisms for plant adaptation to metal toxicity and other stresses involve microRNAs (miRNAs) along with other regulators. miRNAs are 21–24 nt-long non-protein-coding RNAs that regulate plant gene expression at the posttranscriptional level through the transcript cleavage or translation inhibition of their specific mRNA target(s). Generally, miRNA target genes code for transcription factors, stress response proteins, and other proteins that impact the development, growth, and physiology of plants. This mechanism operates through the recruitment of a miRNA-containing effector complex, that includes ARGONAUTE 1 (AGO1) protein, to its target mRNA by base-pairing complementarity (Rogers and Chen, 2013). In addition, miRNAs (23–24 nt-long), loaded to AGO4, are capable of transcriptional gene silencing by triggering

DNA-methylation at some of their target sites (Chellappan et al., 2010; Rogers and Chen, 2013). Several reports have shown the role of miRNAs in the response/adaptation of plants to different abiotic stresses including metal toxicity (reviewed by Gielen et al., 2012; Kraiwesh et al., 2012; Mendoza-Soto et al., 2012; Sunkar et al., 2012; Gupta et al., 2014; Zeng et al., 2014). Specifically, recent studies based in high-throughput sequencing technology, genome-wide analysis of small RNAs and degradome have identified root miRNAs that respond to high Al levels (reviewed by Yang and Chen, 2013; He et al., 2014). These include Alt-responsive miRNAs from rice (*Oryza sativa* sp *indica* and *O. sativa* sp *japonica*; Lima et al., 2011) and from the legumes *Medicago truncatula* (Zhou et al., 2008; Chen et al., 2012) and wild soybean (*Glycine soja*; Zeng et al., 2012). Tobacco (*Nicotiana tabacum*) miRNAs that respond to aluminum oxide (Al_2O_3) nanoparticles have also been reported (Burklew et al., 2012). However, to our knowledge, there are no reports about Alt-responsive miRNAs from nodules of SNF legumes.

In this work we aimed to characterize the response of SNF common bean plants, inoculated with *Rhizobium tropici*, growing in low pH with excess Al and to delineate root and nodule Alt-responsive miRNAs. For comparison we also analyzed phenotypic Alt responses of nitrate-fertilized plants. A miRNA expression profile, based in hybridization of a miRNA macroarray (Valdés-López et al., 2010), was performed to identify Alt-responsive miRNAs in common bean nodules and roots. Expression analysis, based on real-time quantitative reverse transcriptase-PCR (qRT-PCR), was performed for selected Alt-responsive miRNAs and their predicted/validated target mRNAs. Proposed roles of the analyzed miRNA/target nodes in signaling pathways of the nodules/roots from common bean exposed to acidity/Alt are discussed in view of previous studies from other plant species subjected to similar stress. Our work contributes to increase the knowledge about Alt-responsive miRNAs in an agronomical important legume, extensively grown in acidic soils.

Materials and Methods

Plant Material and Growth Conditions

The common bean (*P. vulgaris* L.) Mesoamerican cv. Negro Jamapa 81 was used in this study. Seeds were surface sterilized in 70% (v/v) ethanol for 1 min followed by 10% (v/v) commercial sodium hypochlorite for 10 min and finally rinsed 5–6 times in sterile distilled water where they remained soaking for 12 h. Subsequently seeds were germinated on moist sterile paper towels in the dark at 30°C for 2 days. Germinated seedlings were grown in hydroponic system under controlled environmental conditions as previously described (Valdés-López et al., 2010). The hydroponic trays contained the nutrient solution reported by Franco and Munns (1982b). To induce Alt the pH of the nutrient solution was adjusted to 4.5 using 1 N HCl and it was supplemented with 70 μM $AlCl_3$. For the control treatment, full-nutrient solution without excess Al, the pH was also adjusted to 4.5. Throughout every experiment the pH and volume of the nutrient solution from the hydroponic trays were controlled daily and the nutrient solution was changed every 3–4 days for fresh solution (with or without excess Al).

The $AlCl_3$ concentration (70 μM) used in this work for Alt treatment was selected based in the results of the phenotypic analysis performed in SNF common bean plants subjected to 50, 70, or 100 μM $AlCl_3$ as compared to control treatment. For this experiment common bean plants were grown under symbiotic conditions, as described next; these were harvested for phenotypic analysis 7 days after exposure to a different $AlCl_3$ concentration.

For symbiotic condition, plantlets adapted to grow for 3 days in hydroponic trays with N-free Franco/Munns solution were inoculated with 5 ml of a saturated (over-night) liquid culture of *R. tropici* CIAT 899 (Graham et al., 1994). For Alt stress treatment the nutrient solution was changed at 12 days post-inoculation (dpi), when inoculated plants had already formed nodules, for a solution supplemented with 70 μM $AlCl_3$. These plants were harvested 24 h (13 dpi) or 7 days (19 dpi) after Alt exposure. For control treatment fresh nutrient N-free Franco/Munns nutrient solution was changed at 12 dpi and inoculated plants were harvested at 13 or 19 dpi.

For non-symbiotic condition, full nutrient Franco/Munns solution was used. For Alt-stress treatment, 12 days after planting the nutrient solution was changed for one supplemented with 70 μM $AlCl_3$ and plants were harvested 24 h or 7 days after Alt exposure. For control treatment, fresh nutrient solution with the same composition was changed after 12 days and plants were harvested at 13 or 19 days after planting.

In each experiment the expression of an Al-activated malate transporter (*ALMT*1, Phvul.001G081000[1]) marker gene for Alt (Chandran et al., 2008) was determined by qRT-PCR. In addition, visible morphological changes in roots and nodules from Alt plants as compared to control were checked in every experiment. The decrease in root length, pale pink or whitish colored nodules with a roughened external surface as well as increased expression of the *ALMT1* marker gene indicated the stress-nature of the treatment used.

Both control and stress (symbiotic or fertilized) treatments consisted of three independent plastic trays, with eight seedlings per tray. Three different sets of plants were considered for analysis. From the total plants in each experiment (24) a different number of harvested plants were used for each phenotypic, biochemical or molecular analysis as will be described below.

Phenotypic Analysis

From the total number of plants in each experiment, 10 plants from each treatment (inoculated or fertilized) were harvested at each of the indicated time points for root length and dry weight (DW) determination. The length of the primary root was measured from freshly harvested plants; roots were cut and rinsed with tap water. After eliminating the excess of water with paper towels, each root was placed in a flat surface and the primary root was extended completely to measure it with a ruler. This procedure was done carefully to avoid breakage of the roots. Subsequently each root was dried in an oven at 60°C for 3 days

[1] http://www.phytozome.net, v1.0

and then weighed on an analytical scale to calculate the root DW. In experiments with inoculated plants, nodules were excised from the root before drying the roots. Student's *t*-test was used to analyze the difference in root length and DW between control and Alt stressed plants.

Nitrogenase activity was determined by the acetylene reduction assay (Hardy et al., 1968) in detached nodulated roots from 10 plants form each treatment. Root samples with mature nodules were placed into 20 ml vials, 2 ml of acetylene were injected into the vial to create a 10% acetylene atmosphere. The vials were incubated at room temperature 30 min, 2 ml of the gas of each vial were removed and injected in the gas chromatographer to analyze ethylene concentration. Specific activity is expressed as nmol ethylene h^{-1} g^{-1} nodule DW. Student's *t*-test was used to analyze the difference in nitrogenase activity of Alt stressed plants as compared to control plants.

For the phenotypic analyses described below, the microscopes used were: a light microscope SteREO (Zeiss) or a fluorescence optical microscope Axioskop 2 (Zeiss). From the total number of plants in each experiment, eight individual roots or nodules from each treatment were examined in every staining protocol.

Aluminum accumulation was detected by morin staining following the protocol reported by Tice et al. (1992). Common bean root tips and nodules slices were washed with 5 mM ammonium acetate buffer (pH 5.0) for 20 min, followed by 1 h incubation in 100 mM morin dissolved in the same buffer and finally washed with buffer for 20 min. Green fluorescence from Al-morin complexes was observed at 420 nm excitation and 510 nm emission wavelengths. Production of H_2O_2 ROS was detected after roots incubation for 35 min in a solution containing 200 μM $CaCl_2$ plus 10 μM 6-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (DCF-DA) that emits fluorescence when interacting with H_2O_2 (Jones et al., 2006). ROS-fluorescence was observed at 488 nm excitation and 530 nm emission wavelengths. Lipid peroxidation in excised root and nodules was visualized after staining with Schiff's reagent for 35 min, as reported by Yamamoto et al. (2001). Cell death, assessed by the loss of membrane integrity, was visualized by staining with Evans blue (Yamamoto et al., 2001) during 15 min for roots and 20 min for nodules. Accumulation of callose was determined as reported by Millet et al. (2010) using aniline blue. For this assay entire roots and nodule slices were used. Callose fluorescence was observed immediately under UV (390 nm excitation and 460 nm emission).

Preparation, Hybridization and Data Analysis of miRNA Macroarrays

The approach used to identify miRNAs from nodules of common bean plants from control or Alt treatment was based on the hybridization of miRNA macroarrays as previously reported by Valdés-López et al. (2010). The steps of this protocol are briefly explained next.

Total RNA was isolated from 1 to 2 g frozen nodules of control or Al-treated common bean plants using LiCl precipitation method as reported previously (Valdés-López et al., 2008). Total nodule RNA samples were enriched for miRNAs by using flashPAGE fractionator (Ambion). These samples (hereafter termed 'miRNA samples') were preserved at −80°C until used for miRNA macroarray hybridization.

Forty-two synthetic DNA oligonucleotides (18–24 nts) corresponding to reverse complementary sequences of 42 mature miRNAs families were synthesized. Twenty-three of these DNA oligonucleotides corresponded to conserved miRNAs, 7 to miRNAs from soybean, 9 to miRNAs from common bean, and 3 to miRNAs expressed in *M. truncatula* nodules (Lelandais-Brière et al., 2009). The recently released *P. vulgaris* genome sequence (Schmutz et al., 2014[2]) was analyzed to verify that the precursors of each of the 42-selected miRNA were indeed encoded in the genome. Other DNA oligonucleotide probes complementary to different miRNA families that were used in the miRNA macroarray analysis reported by Valdés-López et al. (2010) were excluded from this analysis because these could not be mapped in the common bean genome. The sequences of the DNA oligonucleotide probes printed in the miRNA macroarray used in this work are provided in Supplementary Table S1. For miRNA macroarray preparation each DNA oligonucleotide probe was manually spotted on 2 cm × 3 cm Amersham Hybond-N^+ membranes, dried at room temperature and UV cross-linked three times.

After hybridization with radiolabeled miRNA samples the macroarray membranes were washed, exposed to a Phosphor Screen System and scanned. Three independent miRNA macroarrays were hybridized with miRNAs isolated from three different plants (biological replicates) of each treatment. The signal intensity of each spot of the miRNA macroarrays was determined using ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA, USA). The signal intensity data were normalized with the average of the signal intensity of the printed miR159 that showed no significant variation across all the conditions tested and therefore has been used by our group in previous works (Naya et al., 2014; Nova-Franco et al., 2015). The normalized data were then used to analyze the level of expression of each miRNA in the nodules from plants grown in control and in metal-toxicity conditions. The raw and normalized signal intensity data from each miRNA macroarray hybridization experiment are shown in Supplementary Table S2.

For analysis of the differential expression of miRNAs in nodules from metal toxicity-stressed plants, the average normalized expression ratios (stressed:control) were obtained and subjected to Student's *t*-test ($p \leq 0.05$).

Semi-quantitative and Real-time Quantitative Reverse Transcriptase-PCR (qRT-PCR) Analysis

Transcript level of the *ALMT1* marker gene in roots under control or Alt stress were analyzed by semi-quantitative RT-PCR that was performed by two-step RT-PCR using polythymine deoxynucleotide primer following the manufacturer instructions (Clontech Laboratories, Inc. Mount View, CA, USA). Annealing temperature was 55°C, 28 cycles were used. Primer oligonucleotide sequences are shown in Supplementary Table S3. Amplified RT-PCR products were resolved on 2% (w/v) agarose

[2]http://www.phytozome.net, v1.0

gels in Tris-acetate-EDTA buffer. The ubiquitin gene (*UBQ*) was included as a control for uniform RT-PCR conditions.

For the quantification of the transcript levels of mature miRNAs, cDNA was synthesized from 500 ng of total RNA the NCode miRNA First-Strand cDNA Synthesis (Invitrogen). Resulting cDNAs were then diluted and used to perform qRT-PCR assays using the Maxima SYBR Green/Fluorescein qPCR master mix (Fermentas, Hanover, MD, USA), following the manufacturer instructions. The transcript levels of selected common bean miRNA target genes were quantified by the one-step assay using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA, USA). Each qRT-PCR reaction contained 100 ng of RNA template, previously treated with DNase (Qiagen, Hilden, Germany). Both for mature miRNAs and for target genes transcript level determinations, qPCR reactions were run in a 96-well format with the iQ5 Real-Time PCR Detection System and iQ5 Optical System Software (Bio-Rad) with settings of 10 min at 50°C (cDNA synthesis), 5 min at 95°C (iScript reverse transcriptase inactivation), followed by 40 cycles for PCR cycling and detection of 30 s at 55°C. Supplementary Table S3 provides the sequences of the oligonucleotide primers used for qRT-PCR amplification of each gene.

Three biological replicates with two technical replicates each were carried out for the determination of transcript level of each gene or miRNA, RNA was extracted from differing sets of plants grown under similar treatment (control or Alt). Relative transcript levels for each sample were obtained using the 'comparative C_t method'. The threshold cycle (C_t) value obtained after each reaction was normalized to the C_t value of miR159 for miRNA levels or to the C_t value of the elongation factor 1 (EF1) gene (Phvul.004G060000) for target gene levels; these reference genes were constant across the conditions (Supplementary Table S2). The relative expression level was obtained by calculating the $\Delta\Delta C_t$ values for the stress conditions used and the normalized C_t value (ΔC_t) for the controls. The normalized fold expression levels were subjected to Student's *t*-test ($p \leq 0.05$).

Results

Response of Common Bean Plants to Al Toxicity

The objective of this work was to characterize the response of SNF common bean plants to acidic/Al-toxicity stress, aiming to describe the symptoms present as well as the miRNAs differentially expressed in active nodules from plants exposed to Alt, something that is yet poorly documented for this or other legumes. To achieve our objective, our experimental design took into consideration previous knowledge about the negative effect of low pH on rhizobia root colonization/infection and nodule development/function in common bean (Franco and Munns, 1982a; Vassileva et al., 1997). Such negative effects are related to the acid sensitivity of free-living rhizobia (Graham et al., 1994; O'Hara and Glen, 1994). Our experimental design, based on that reported several years ago by Franco and Munns (1982a), has been used by our group to describe the miRNA expression profile from previously developed nodules of SNF common bean plants

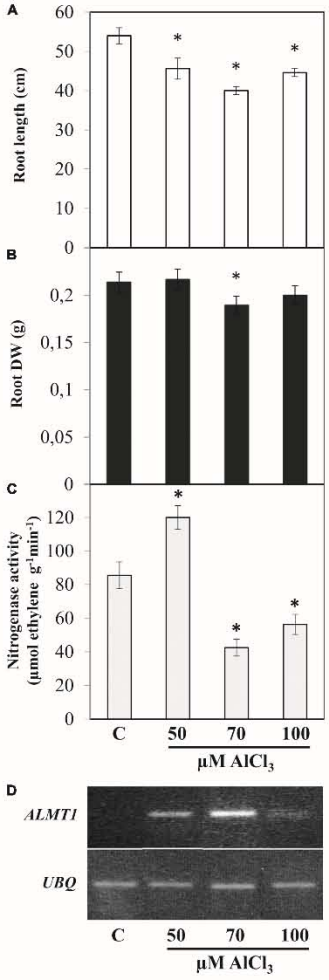

FIGURE 1 | Effect of different $AlCl_3$ concentrations on symbiotic nitrogen-fixing common bean plants. The indicated $AlCl_3$ concentration was added to (12 dpi) *Rhizobium tropici*-inoculated plants with active nodules for 7 days. C: control condition without $AlCl_3$. Values of root length **(A)**, root dry weight (DW) **(B)** or nitrogenase specific activity **(C)**, as determined by the acetylene reduction assay, are mean + SE from 10 different plants (biological replicates) per treatment. Student's *t*-test was used to analyze the difference in each parameter as compared to control treatments. Columns marked with star (*) represent significantly different means according to the statistical analysis ($p \leq 0.05$). **(D)** The expression of the Alt-stress marker gene *ALMT1* was evaluated by semi-quantitative RT-PCR from roots of plants inoculated plants grown in each treatment, as indicated. The ubiquitin gene (*UBQ*) was included as a control for uniform RT-PCR conditions. Shown is a representative gel from a total of three experiments.

that were exposed to a nutrient deficiency, acidity or Mn toxicity. In short, plantlets adapted to grow in hydroponic conditions were inoculated with *R. tropici* CIAT 899 that is acid tolerant (Graham et al., 1994), when functional nodules were formed stress was imposed by changing the nutrient solution to one containing 70 μM $AlCl_3$. For the control treatment, another set of nodulated plants continued to grow in N-free nutrient solution. For both treatments the pH of the solution was adjusted to 4.5 and was controlled throughout the experiment, this allowed to separate the effect of Al from that of low pH.

The degree of toxic effect induced by a certain Al concentration varies considerably depending on the plant species/genotype, the experimental conditions and other factors (Kochian et al., 2005). Therefore initially we tested different $AlCl_3$ concentration in order to select the adequate treatment for SNF common bean plants to be used this work. After 12 dpi *R. tropici*-nodulated common bean plants were transferred to nutrient solution supplemented with 50, 70, or 100 μM $AlCl_3$ (pH 4.5) and were harvested 7 days after Al exposure for phenotypic analysis (**Figure 1**). As compared to control condition (N-free nutrient solution, pH 4.5), SNF common bean plants exposed to 70 μM $AlCl_3$ showed highest reduction in root length (30%, **Figure 1A**) and in root DW (14%, **Figure 1B**) as compared to plants in the other Al-stress treatments tested. In addition, the nitrogenase activity (acetylene reduction assay) from nodules under 70 μM $AlCl_3$ was the lowest (50%, **Figure 1C**). These data are in agreement with the expression level of *ALMT1* marker gene observed in roots of plants from the different treatments; it was highest in roots from plants under 70 μM $AlCl_3$ treatment (**Figure 1D**). On this basis, for this work the selected $AlCl_3$ concentration for Alt in common bean was 70 μM.

Due to the genotypic variability and diverse experimental conditions it is difficult to reach a consensus on the timing for Alt in plants; different responses have been observed at early or at late exposure to Al-stress (Kochian et al., 2005). In this work we analyzed common bean response to Alt (70 μM) at two periods of Al exposure. The long period selected for Alt-response analysis was 7 days, based in our previous work (Valdés-López et al., 2010) and we included 24 h exposure for analysis of an earlier response. **Figure 2A** shows the data of the phenotypic analysis performed in SNF bean plants exposed to Alt treatment (24 h or 7 days) as compared to control plants. No significant change was observed in the leaf area or the nodule DW from SNF plants under Alt treatments (data not shown). SNF plants exposed for 7 days to Alt showed a decreased in root DW (50%) and root length (20%), while plants from 24 h treatment did not show changes in these parameters. However, as evidenced by nitrogenase activity (acetylene reduction assay) values, both Alt treatments affected nodule function since a 43 and 62% reduction at 24 h and 7 days, respectively, was observed.

We compared the phenotypic analysis of SNF common bean plants with full-nutrient fertilized common bean plants subjected to Alt for short or long periods. Similar condition as those for nodulated plants were used, Alt was added for 24 h or 7 days to roots of plants pre-grown for 12 days in full-nutrient solution. No alteration in leaf area was observed in all the treatments analyzed (data not shown). In Al-treated fertilized plants a decrease in root DW (28%) was observed only at 7 days treatment, while a decrease in root length was observed both at 24 h (6%) and 7 days (25%) treatments (**Figure 2B**).

Following the characterization of the response of SNF common bean to Alt, we performed a histological analysis, in

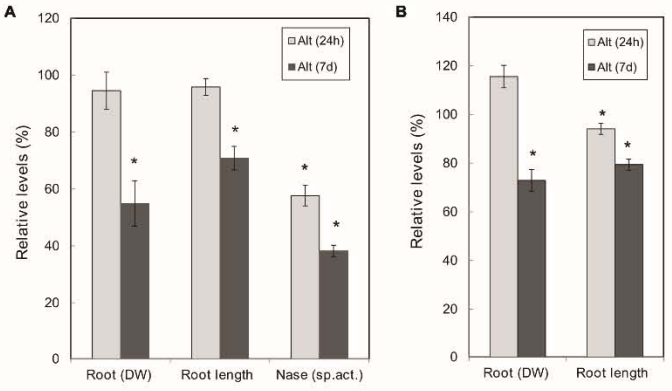

FIGURE 2 | Effect of Al toxicity on common bean plants. High Al (70 μM $AlCl_3$) was added to (12 dpi) *R. tropici*-inoculated plants with active nodules **(A)** or to (12 days) fertilized plants **(B)**, for 24 h or 7 days as indicated. Values are expressed relative to those from nodulated **(A)** or fertilized **(B)** plants grown in control condition (100%). Nitrogenase specific activity, as determined by the acetylene reduction assay, is expressed per nodule DW. Values are the mean ± SE for ten biological replicates from different plant sets. Student's *t*-test was used to analyze the difference in each parameter between plants grown under stress vs. control treatments. Columns marked with star (*) represent significantly different means according to the statistical analysis ($p \leq 0.05$).

both roots and nodules, to observe symptoms known to be specific and characteristic of roots from different plants exposed to excess Al (Kochian et al., 2005; Vitorello et al., 2005). This was done after long period (7 days) exposure to Alt because this treatment resulted in the highest effect on SNF plants (**Figure 2A**). Fluorescence resulting from accumulation of Al, after morin staining, was observed in the root tips and in the infection zone of mature nodules of plants exposed to Alt, while no fluorescence was observed in organs from control plants (**Figure 3A**). ROS (H_2O_2) accumulation, evidenced as fluorescence after DCF-DA treatment, was high in the Alt roots elongation zone as compared to roots from control plants (**Figure 3B**). This test was not useful to detect ROS from nodules since DCF-DA could not penetrate into the nodules and no fluorescence could be observed. Callose-fluorescence was observed in the elongation zone of bean roots (**Figure 3C**). In nodules, the callose-fluorescence was observed mainly on the outer layer (**Figure 3C**). Peroxidative damage of membrane lipids (lipoperoxidation) due to the stress-related increase in high toxic ROS is often associated with Alt (Cakmak and Horst, 1991). The Schiff's reagent was used to visualize aldehydes derived from lipoperoxidation. In roots from Alt bean plants an intense pink staining was observed mainly in the elongation zone and in the whole infection zone of stressed nodules (**Figure 3D**). An oxidative damage leads to a disturbance of cellular homeostasis that could result in cell death thus we used Evan's blue staining to assess cell death in Alt roots and nodules. Cell death was evident in the tips and the elongation zone of roots of stressed bean plants (**Figure 3E**). In Al-stressed nodules we observed a build up of tissue that gave a roughened texture to the external surface. Evan's staining of entire nodules revealed that cell death could be occurring in the rough external layer (**Figure 3E**).

miRNAs Expression Profile in Nodules of Common Bean Plants Under Alt

Recent studies have identified miRNAs that are differentially expressed in tissues of plants exposed to Alt; to our knowledge, these do not include legume nodules (Zhou et al., 2008; Lima et al., 2011; Burklew et al., 2012; Chen et al., 2012; Zeng et al., 2012). Alt-responsive miRNAs are likely to play important roles in the regulation of plant response/adaptation to this stress. In this work we aimed to identify miRNAs from common bean nodules exposed to high Alt for 7 days; in this treatment SNF bean plants showed major phenotypic alterations (**Figure 2**).

The miRNAs expression profile analysis from Alt nodules was performed through hybridization of a miRNA macroarray; the membranes used contained 42 DNA oligonucleotide probes (Supplementary Table S1) complementary to genome-mapped miRNAs that are expressed in different tissues of common bean plants (Peláez et al., 2012).

The data on normalized expression level (stressed:control) of each of the 42 miRNA families analyzed through macroarray hybridization revealed that 28 miRNAs were differentially regulated in Alt-stressed common bean nodules; half of these were up-regulated (**Table 1**). Among the up-regulated miRNAs we found 10 conserved miRNAS, from these miR164 and miR396 showed the highest response; while eight conserved miRNAs were down-regulated (**Table 1**). Also we found 10 Alt-responsive miRNAs that have been identified in one or more legume species (common bean, soybean and/or *M. trucatula*), thus putative legume-specific miRNA families, six of these were down-regulated (**Table 1**).

Expression Analysis of Selected Metal Alt-Responsive miRNAs and their Target Genes

We selected eight Alt-responsive miRNAs from common bean nodules, including both up-regulated and down-regulated examples, to validate the expression rates obtained in the miRNA macroarray experiment (**Table 1**) using the alternative method of qRT-PCR. Besides validating the expression of selected miRNAs on nodules from SNF plants exposed for long period to Al (**Table 1**) we extended the analysis to nodules under Alt-stress for short period, and also to roots from fertilized plants exposed to Al for long or short periods (**Figures 4** and **5**).

To gain insight into specific roles of miRNAs in common bean response to Al stress, we included a qRT-PCR expression analysis of the genes targeted by the selected miRNAs (**Figures 4** and **5**). Very few common bean miRNAs target genes have been experimentally validated (Arenas-Huertero et al., 2009); we considered this an important issue for the selection of miRNAs/target nodes to be used for expression analysis in this work. So six, out of eight, miRNAs that were selected for expression analysis in this work have a validated target gene in common bean (Arenas-Huertero et al., 2009); these include four up-regulated and two down-regulated miRNAs according to data from macroarrays (**Table 1**). Each miRNA may target several genes, from the same or from different gene families (Kraiwesh et al., 2012; Sunkar et al., 2012; Rogers and Chen, 2013). In order to define this for common bean miRNAs, further genome-wide analysis based in the recently published genome sequence (Schmutz et al., 2014) as well as experimental validation for predicted targets is required. However, in this work we focused in the expression analysis of the only validated (or predicted) target gene for each selected miRNA (Arenas-Huertero et al., 2009; Valdés-López et al., 2010). The selected up-regulated miRNAs and their corresponding validated target genes (Arenas-Huertero et al., 2009) are: miR164/NAC1 (NAM/ATAF/CUC transcription factor), miR170/SCL (SCARECROW-like protein transcription factor), miR393/TIR1 (TRANSPORT INHIBITOR RESPONSE 1-like protein) and miR396/GRL1 (GROWTH-REGULATING FACTOR-like protein). The selected down-regulated miRNAs and their corresponding validated target (Arenas-Huertero et al., 2009; Naya et al., 2014) are: miR157/SPL (SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE) and miR398/CSD1 (Cu/Zn SUPEROXIDE DISMUTASE). We also selected miR169 that showed the highest value for down-regulation in Alt-stressed common bean nodules (**Table 1**). This was the first miRNA whose role in nodule development was demonstrated in *M. truncatula*; it targets the transcription factor NF-YA1 (NUCLEAR FACTOR YA1, previously called HAP2) was experimentally validated in *M. truncatula* and in soybean and it was predicted by bioinformatics analysis for

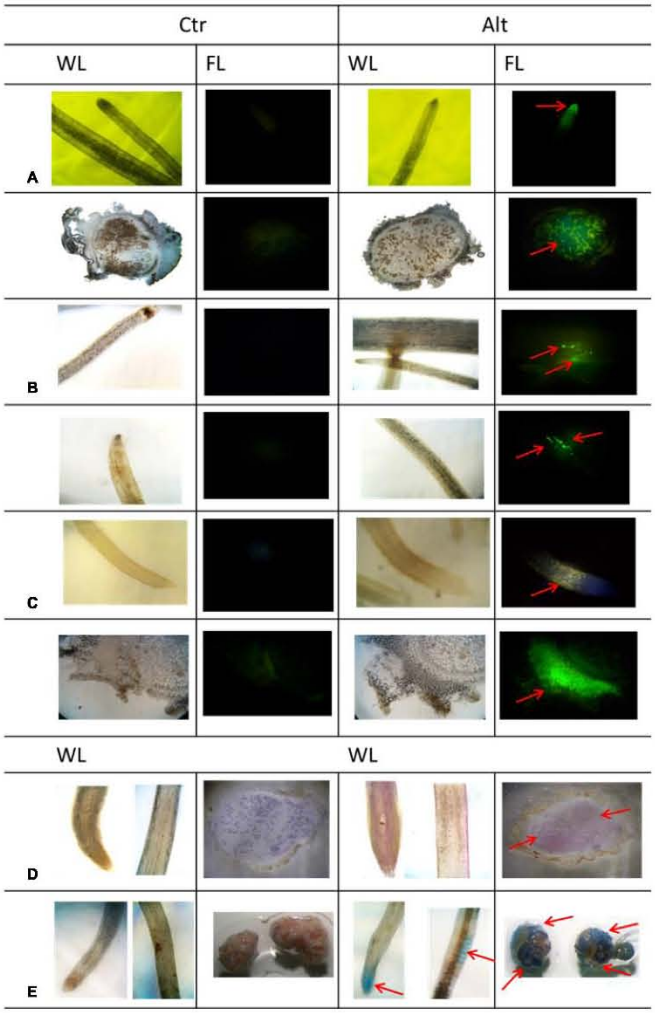

FIGURE 3 | Histological analysis of Al toxicity response in roots and mature nodules of *R. tropici*-inoculated common bean plants. SNF common bean plants were grown in control (Ctr) or in Al- toxicity (Alt) conditions. **(A)** Accumulation of Al evidenced by Al-morin fluorescence. Magnification = 10X, for roots and nodules **(B)** ROS (H_2O_2) production detected by DCF-DA fluorescence. Magnification = 10X **(C)** Callose distribution evidenced by fluorescence emitted after aniline blue staining. Magnification = 10X for roots and 20X for nodules. Magnification = 10X for roots and 8X for nodules **(D)** Lipoperoxidation visualized after Schiff's reagent staining **(E)** Cell death visualized after Evans blue staining. Magnification = 10X for roots and 4X for nodules. Red arrows point out the specific symptoms commented in the text. WL, white light, FL, fluorescent light. Each histological analysis was repeated in eight roots or nodule samples from different plants grown in control or Alt treatment, representative images are shown.

common bean (Combier et al., 2006; Arenas-Huertero et al., 2009; Song et al., 2011). In addition, we selected pvu-miR1511, up-regulated in Alt nodules (**Table 1**), its predicted target in common bean target gene is SP1L (SPIRAL-like protein, a microtubule-associated protein; Arenas-Huertero et al., 2009); this miRNA has been found in the legumes *M. truncatula*

and soybean (Lelandais-Brière et al., 2009; Song et al., 2011; Formey et al., 2014) but its regulatory function remains unknown.

Figure 4 shows the data of the expression ratios of the selected miRNAs and their target genes in active nodules that were exposed 7 days to Alt. The tendency (up- or down-regulation) in the response to Alt of each miRNA tested using qRT-PCR approach was similar to that shown **Table 1**, thus validating the miRNA macroarray results. However, there was variation in the expression ratio values obtained from macroarrays as compared to those from qRT-PCR analysis (**Figure 4**, **Table 1**) that could be attributable to different sensitivities of the two methods. In every case, the expression ratio of the target gene showed the expected inverse correlation with that of its miRNA (**Figure 4**). The respective target genes of the miRNAs miR164, miR170, miR393, miR396, and pvu-miR1511 were down-regulated pointing to the miRNA-induced target cleavage (**Figure 4**).

We also analyzed the expression of the selected miRNA/target nodes in developed roots from fertilized plants subjected to Alt for 7 days (**Figure 4**). As shown in **Figure 4**, miR164, miR170, and miR1511 were up-regulated while miR157, miR169, and miR398 were down-regulated in 7 days Alt roots. The target genes of these Alt root responsive miRNAs showed an inverse correlation with that of their miRNA (**Figure 4**). miR393 and miR396 did not show a significant response in Alt roots (**Figure 4**).

To assess if the observed miRNA response was characteristic of a long-time exposure to Alt (**Figure 4**) we compared it to that of short Alt exposure thus determining the expression of the selected miRNA/target nodes in nodules and roots exposed to high Al for 24 h (**Figure 5**). In 24 h Alt nodules miR170, miR396, and pvu-miR1511 were significantly up-regulated, miR157 and miR398 were significantly down-regulated and their target genes showed the respective inverse correlation; whereas miR164, miR393, and miR169 did not show a significant response (**Figure 5**). The expression analysis of selected miRNA/target nodes in 24 h Alt roots from fertilized plants revealed up-regulation of miR170, miR393, and miR396 and down-regulation of miR169 and miR398, with the corresponding inverse correlation of their target genes; whereas miR164, miR157, and pvu-miR1511 did not show a significant response (**Figure 5**)

Discussion

In this work we report the negative effects of Alt to SNF and fertilized common bean plants, grown hydroponically in acidic nutrient solution supplemented with 70 μM $AlCl_3$. This $AlCl_3$ concentration is within the range of those found in acidic soils and those that have been previously used for Alt-stress experiments in common bean and in soybean (Franco and Munns, 1982a; Menzies et al., 1994; Rangel et al., 2007; Yang et al., 2009; Kopittke et al., 2015). In this work we used the black-seeded "Negro Jamapa 81" cultivar and *R. tropici* CIAT 899, an acid pH tolerant rhizobia strain (Graham et al., 1994), as inoculant. Though Franco and Munns (1982a) reported that black seed common bean varieties are less sensitive to acid soils with high Al concentration as compared to non-black seed varieties, Blair et al. (2009) reported that Mesoamerican common bean genotypes are less resistant to Al than Andean gene pools. The later report, aimed to identify Al resistant Andean common bean genotypes, analyzed 36 genotypes including 11 from the Mesoamerican gene pool but Negro Jamapa was not reported in this analysis (Blair et al., 2009). To our knowledge the degree of Al/acid soil resistance of the Negro Jamapa 81 cultivar remains to be analyzed, though this is out of the scope of our present work. The acid soils/Al stress resistance of both symbionts is likely to influence the survival/growth of common bean plants in the treatment used and also the miRNA response. Interestingly, future research may define if the Alt responsive common bean miRNAs identified account

TABLE 1 | MicroRNA (miRNA) expression in nodules of common bean plants grown under control or Alt condition.

miRNA	Expression Ratio (±SE)	*P*-value
miR 160	1.91 (±0.01)	3.60E-05
miR 164	5.35 (±0.03)	8.06E-06
miR 165	1.64 (±0.02)	0.00035
miR 166	1.94 (±0.06)	0.0002
miR 170	5.70 (±0.52)	0.00104
miR 172	1.63 (±0.10)	0.00216
miR 390	2.57 (±0.01)	3.38E-06
miR 393	2.12 (±0.08)	0.00464
miR 395	2.02 (±0.01)	4.42E-05
miR 396	5.98 (±0.12)	0.00156
pvu-miR159.2	3.88 (±0.17)	0.0001
pvu-miR1509	2.13 (±0.01)	0.00088
pvu-miR1511	2.89 (±0.01)	9.53E-06
pvu-miR 2118	2.21 (±0.01)	7.95E-05
miR 156	−1.16 (±0.009)	0.00387
miR 157	−3.57 (±0.004)	1.57E-05
miR 167	−1.38 (±0.05)	0.04902
miR 169	−10.0 (±0.003)	8.10E-06
miR 319	−2.30 (±0.01)	0.00022
miR 398	−1.45 (±0.01)	0.04504
miR 399	−2.56 (±0.03)	0.00120
miR 408	−1.29 (±0.008)	0.02278
pvu-miR1514a	−1.66 (±0.001)	0.00515
pvu-miR1515	−3.70 (±0.001)	0.01827
pvu-miR2119	−2.63 (±0.03)	0.01199
gma-miR1521	−2.00 (±0.006)	7.00E-05
gma-miR1534	−2.56 (±0.005)	0.00415
mtr-miR2586	−1.60 (±0.005)	0.015564

High aluminum was added at 12 dpi for 7 days. The significant expression ratios (Alt:control) are the average of normalized signal intensity values from three biological replicates of miRNA-macroarrays. Names of non-conserved miRNAs, reported previously (Arenas-Huertero et al., 2009; Lelandais-Brière et al., 2009; Valdés-López et al., 2010), include abbreviation of the legume species where these were first annotated: pvu, Phaseolus vulgaris, gma, Glycine max, and mtr, Medicago truncatula. For ratios lower than 1 (miRNAs down-regulated in stress), the inverse of the ratio was estimated and the sign was changed.

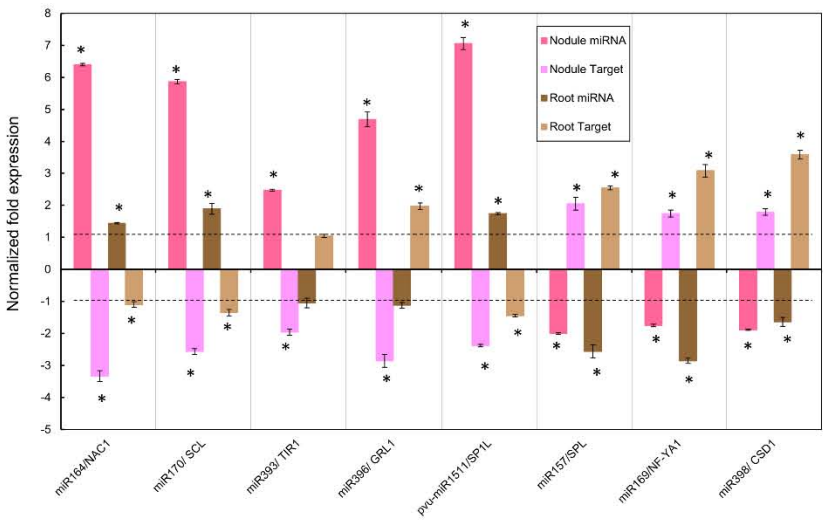

FIGURE 4 | Normalized fold expression levels of selected miRNA/target mRNA nodes in fertilized roots and in SNF nodules of common bean plants under Alt for long period. High Al was added to (12 dpi) *R. tropici*-inoculated plants with active young nodules or to (12 days) fertilized plants for 7 days before harvesting. miRNAs and target genes expression levels were determined by qRT-PCR in nodules of inoculated plants or in roots of fertilized plants. Values represent the normalized expression ratios (stress:control) given as the average of three biological replicates. For ratios lower than 1, the inverse of the ratio was estimated and the sign was changed. Columns marked with star (*) represent significantly different means according to the statistical analysis ($p \leq 0.05$). NAC1, NAM/ATAF/CUC transcription factor; SCL, SCARECROW-like protein; TIR1, TRANSPORT INHIBITOR RESPONSE 1-like protein; GRL1, GROWTH-REGULATING FACTOR 1; SP1L, SPIRAL-like protein 1; SPL, SQUAMOSA PROMOTER-BINDING protein-like; NF-YA1, NUCLEAR FACTOR YA1; CSD1, Cu/Zn SUPEROXIDE DISMUTASE.

for general responses to the stress or if the response may vary among varieties with different adaptation/tolerance to Alt in acid soils.

A decrease in root length was the main and primary effect observed in common bean plants under Alt. After a short period under Alt common bean plants showed *ca.* 6% decrease in root length, equivalent to 2 cm shorter roots as compared to roots from control plants. This result is in agreement with previous works in common bean showing that the transition and the elongation zones of the root are the major targets of Al injury resulting in a rapid inhibition of root elongation (Rangel et al., 2007; Yang et al., 2009). After long period of Al exposure higher decrease in root length was observed, in inoculated and in fertilized plants. These results are in agreement with previous studies indicating that nodulated legumes are more sensitive to Al and Mn toxicity than plants fertilized with mineral N (Hungria and Vargas, 2000). Alt-stressed common bean nodules showed a decrease in nitrogenase activity together with accumulation of Al in their infected zone, thus indicating the presence of high Al in both symbionts. Bacteria under excess Al utilize Fe transport systems for Al uptake that interfere with their ability to capture Fe, an essential micronutrient required for rhizobial nitrogenase activity in rhizobia (Davis et al., 1971; Rogers et al., 2001). Species like *Sinorhizobium meliloti* and *Bradyrhizobium* growing *ex planta* are extremely sensitive to Al since it affects the enzymatic activities for nitrate and nitrite reduction, nitrogenase and uptake hydrogenase (Arora et al., 2010).

The Alt stressed bean roots and nodules showed characteristic Al-stress symptoms observed in roots from different plants such as accumulation of ROS and callose as well as lipoperoxidation. Noticeably, callose and H_2O_2 (ROS) accumulation co-localized in the bean root elongation zone similar as in Alt maize plants (Jones et al., 2006). Al-stressed maize root cells induce callose accumulation and cell wall/plasma membrane rigidification as well as an oxidative burst (ROS) with increasing cytoplasmic Ca^{2+} that leads to activation of the callose synthase enzyme (Jones et al., 2006). Though callose has been observed, in the cell walls of some yeasts, fungi and bacteria (Stone and Clarke, 1992) we did not observe callose-fluorescence in the nodule infection zone where *R. tropici* bacteroids reside, but in the external layers of Alt nodules. We propose that this may function as a protective barrier against Al uptake. It has been reported that callose accumulation can prevent higher uptake of Al and other metals (Van de Venter and Currier, 1977; Wissemeier and Horst, 1992) and also it may prevent pathogen infection (Kohler et al., 2002).

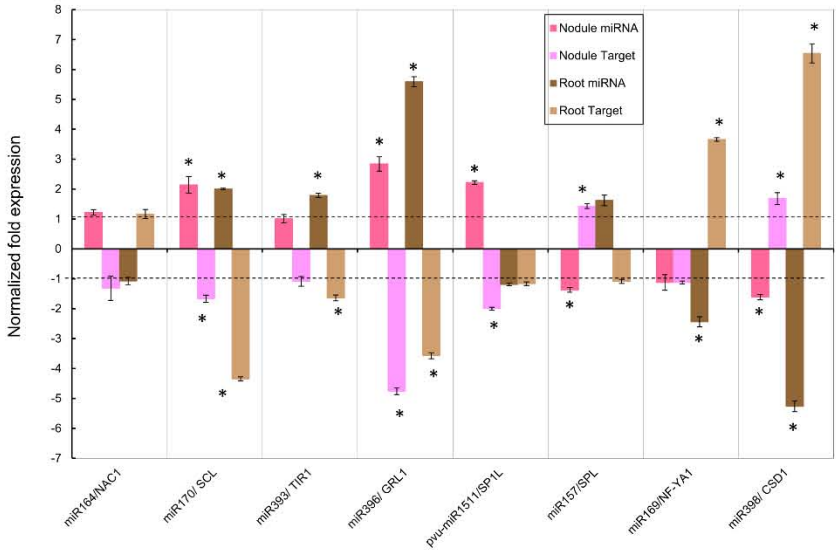

FIGURE 5 | Normalized fold expression levels of selected miRNAs/target mRNA nodes in fertilized roots and in SNF nodules of common bean plants under Alt for short period. High Al was added to (12 dpi) *R. tropici*-inoculated plants with active young nodules or to (12 days) fertilized plants for 24 h before harvesting. miRNAs and target genes expression levels were determined by qRT-PCR in nodules of inoculated plants or in roots of fertilized plants. Values represent the normalized expression ratios (stress:control) given as the average of three biological replicates. For ratios lower than 1, the inverse of the ratio was estimated and the sign was changed. Columns marked with star (*) represent significantly different means according to the statistical analysis ($p \leq 0.05$). See **Figure 4** for target genes description.

The identification of Alt-responsive miRNAs is a first step toward unraveling their regulatory role for plants adaptation/defense to this stress. To our knowledge, there is only one report about nutrient-deficiency and Mn-toxicity responsive miRNAs from common bean nodules (Valdés-López et al., 2010). In the present work we identified 28 Alt-responsive miRNAs in common bean nodules, using a miRNA macroarray hybridization approach proven to be inexpensive and suitable for this type of analysis (Valdés-López et al., 2010). However, in this work the miRNA macroarray design was improved by including only miRNAs encoded by and expressed from the common bean genome (Peláez et al., 2012; Schmutz et al., 2014), thus avoiding detecting non-specific hybridization signals that we now interpret as false positives.

Expression profile of Al-stress responsive miRNAs has been reported for wild soybean, *M. truncatula*, rice and tobacco (Zhou et al., 2008; Lima et al., 2011; Burklew et al., 2012; Chen et al., 2012; Zeng et al., 2012; Yang and Chen, 2013; He et al., 2014). We did a comparative analysis of common bean miRNA families identified in this work with those found in other plant species subjected to similar Al-stress. As shown in **Figure 6**, 19 out of 28 miRNAs identified for common bean nodules are shared by one of more of the Al-stressed plant species; the rest miRNA families only identified in Alt common bean include 6 that have been found only in legumes (common bean, soybean and/or *M. truncatula*) and could represent family- or species-specific miRNAs (Song et al., 2011; Peláez et al., 2012; Formey et al., 2014). We identified three of these miRNA families (pvu-miR1509, pvu-miR2118, and pvu-mIR1514) only in Al-stressed common bean and wild soybean (*G. soja*; **Figure 6**), the latter is the ancestor of the domesticated soybean (*Glycine max*), a widely grown legume crop that is phylogenetically related to common bean (Zeng et al., 2012; Schmutz et al., 2014). *M. truncatula*, rice and tobacco shared 9, 10 and 9 Alt-responsive conserved miRNAs with common bean though several of these showed different trend (up- or down-regulation) as compared to that in common bean. It should be noted that Alt-responsive miRNAs were identified in different tissues: nodules for common bean, roots for wild soybean and rice, root tips for *M. truncatula* and seedling for tobacco (Lima et al., 2011; Burklew et al., 2012; Chen et al., 2012; Zeng et al., 2012). The commonalities among Alt-responsive miRNA families from different species indicate their role in the regulation of general relevant plant responses to this stress, while differences among the response on miRNA families may be related to specific roles in a certain plant tissue and/or species.

In this work we confirmed the macroarray results of eight selected Alt responsive miRNAs through the qRT-PCR

expression analysis. This was complemented with the selected miRNA expression analysis in nodules at short period and roots at long and short periods under Alt. The action of miRNAs is exerted through the silencing of their corresponding target gene(s). To this end, we analyzed the expression level of the genes targeted by the selected Alt responsive nodule miRNAs. These showed the expected opposite trend to the miRNA expression, thus indicated the miRNA-induced target cleavage. The miRNA/target nodes analyzed in this work are known to be involved in relevant signaling pathways that regulate developmental processes or stress responses in different plants and thus allow us to propose their role in the response to Alt of common bean roots and nodules.

In general miRNA response in Al-stressed roots was more significant at short period, something that is in agreement with the roots sensing Alt effects in first place and showing earliest stress responses (Kochian et al., 2005; Vitorello et al., 2005). In this regard we observed that miR393 and miR396 did not respond in 7 days fertilized roots whereas these were up-regulated at short period, while miR169 and miR398 were down-regulated at a higher level in short- than in long-period stress. By contrast, generally a higher miRNA nodule response was observed at 7 days Alt, so perhaps miRNAs are have more important roles in trying to maintain nodule function even in prolonged Al-stress. Notably, nitrogenase activity was already considerably (43%) affected after 24 h Alt treatment so it seems that common bean nodule dysfunction precedes the turn-on of Alt stress signaling pathways related to miRNA differential expression. Although all the selected miRNA have a significant respond in 7 days Alt nodules, half of these -miR164, miR169, miR393, and pvu-miR1511- did not respond at short period of Al exposure.

Our analysis allows comparing miRNA responses to Al exposure in roots from fertilized plants vs. nodules from SNF plants. At long Al exposure, miR164, miR170 and miR1511 were highly up-regulated in nodules and showed the same trend in roots though to lower levels. Their corresponding target genes showed down-regulation thus indicating an important role for silencing NAC1 and SCL TF -involved auxin signaling and developmental processes, respectively- in both tissues under Alt. Similarly miR157, miR169, and miR398 were down-regulated in nodules and in roots with the corresponding up-regulation of their target genes (SPL and NF-YA1 TF and CDS1, respectively) that may have a relevant role in coping with Al stress in both organs through transcriptional regulation or ROS detoxification. By contrast at long Al exposure miR393 and miR396 showed a tendency for down-regulation in fertilized roots, although this was not statistically significant, while in nodules these miRNAs showed high up-regulation with the corresponding target gene silencing; thus indicating a relevant and more specific role in SNF Al stressed nodules.

We observed that miR164 and miR393 were highly up-regulated and their corresponding target genes NAC1 (transcription factor) and TIR1 (auxin receptor) were down-regulated in nodules exposed to Al for long period, while in 7 days fertilized roots miR164 showed a minor up-regulation and miR393 did not respond. miR164 was also reported as

miRNA	Pv	Gs	Mt	Os	Nt
miR 160					
miR 164					
miR 166					
miR 172					
miR 390					
miR 393					
miR 395					
miR 396					
pvu-miR1509					
pvu-miR 2118					
miR 156					
miR 157					
miR 167					
miR 169					
miR 319					
miR 398					
miR 399					
miR 408					
pvu-miR1514a					

FIGURE 6 | Alt-responsive microRNAs from several plant species. Pv, *Phaseolus vulgaris*, Gs, *Glycine soja*, Mt, *Medicago truncatula*, and Nt, *Nicotiana tabacum*. Green, up-regulation and red, down-regulation. References: Zhou et al. (2008), Lima et al. (2011), Burklew et al. (2012), Chen et al. (2012), and Zeng et al. (2012).

Alt-responsive in wild soybean and miR393 in *M. truncatula* and in rice (**Figure 6**). In *Arabidopsis*, the miR164/NAC1 and miR393/TIR1 nodes are involved in the auxin-signaling pathway that controls lateral root development (Guo et al., 2005; Navarro et al., 2006; Chen et al., 2011). In legumes the auxin/cytokinin ratio is strictly controlled and plays an important role in nodule development during the legume-rhizobia symbiosis (Ferguson and Mathesius, 2003). We propose that the auxin signaling pathway is a relevant component in the signal transduction for the response of common bean nodules to Al-stress. In addition, *Arabidopsis* miR393 and its target AFB3 (another auxin receptor) is a unique N-responsive node that regulates auxin response and controls root system architecture (lateral root formation) in response to N availability (Vidal et al., 2010). Though AFB3 has not been validated as miR393 target in legumes, we found AFB3 orthologs from soybean (Gmax19g27280) and from common bean (Phvul.001G087000) with putative miR393 binding sites within their coding region. Diminished nitrogenase activity in Alt common bean nodules would result in low N content, something that may regulate the expression of miR393 as observed.

Common bean roots and nodules under Alt for short and long periods showed up-regulation of miR170 and the corresponding down-regulation of its target SCL (transcription factor). The miR170/SCL node has been involved the gibberellin signaling

pathway. Gibberellins promote cell elongation involved in root growth (Inada et al., 2000) and regulate lateral root formation through interactions with auxins and other hormones (Gou et al., 2010). In *Allium sativum* root tip cells the presence of gibberellins has been related with the restoration of lipid peroxidation and genotoxicity by metals such as cadmium (Celik et al., 2008). In nodules gibberellins biosynthesis is up-regulated during later stages of nodulation, these are required for proper mature nodule structure (Hayashi et al., 2014). We propose that the role of miR170/SCL and gibberellin signaling is relevant for the response of common bean roots/nodules to Al-stressed and it may be related to the regulation of characteristic responses such as root growth and lipid peroxidation. Notable, miR170 was not reported as Alt-responsive in wild soybean or *M. truncatula* roots (Chen et al., 2012; Zeng et al., 2012), thus indicating that its regulatory role is rather specific to mature nodule structure/function in common bean and maybe in other legumes.

We observed that miR398 was down-regulated and its target gene CSD was up-regulated in common bean roots and nodules under Alt for short and long periods. Similar response for miR398 was observed for *M. truncatula* and rice roots (**Figure 6**). miRNA398 was the first miRNA described as oxidative stress responsive in plants (Sunkar et al., 2006). Oxidative stress generated upon exposure to toxic concentrations of metals like copper (Cu), suppresses *Arabidopsis* miR398 expression that is essential for the accumulation of CSD1 and CSD2 required for detoxification of ROS (Sunkar et al., 2006). In common bean miR398/CSD1 node responds to oxidative stress and high ROS production resulting from Cu toxicity and from biotic interactions (Naya et al., 2014). It is known that Alt generates oxidative stress and ROS production in plants (Yamamoto et al., 2001; Kochian et al., 2005; Navascués et al., 2011), here we evidenced ROS increase and lipoperoxidation in Alt common bean nodules; therefore the role of miR398/CSD in Al-stress seems to be part of the response to oxidative stress generated by different stimuli in most plant species.

Data presented in this work form a basis for further analysis leading to demonstration of specific roles of candidate common bean miRNAs in the response of common bean roots/nodules to Al-stress, using genetic approaches. Though common bean mutant collections are not yet available, reverse genetic approaches in composite bean plants, with transgenic hairy roots and nodules offer a suitable alternative. Our group in fact demonstrated the role of miR399 in phosphorus deficient bean roots using a reverse genetic approach (Valdés-López et al., 2008). Studies aimed at analysis of specific miRNA functions in response to Alt stress in SNF bean plants are in progress.

Author Contributions

AM-S conceived and performed experiments, interpreted data and contributed to the drafting of the manuscript. LN conceived experiments, gave experimental advice and contributed to the drafting of the manuscript. AL performed experiments. GH conceived and supervised the whole project and wrote the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00587

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3.2 Toxicidad por Cobre en plantas de frijol en simbiosis con rhizobia

3.2.1 Características fenotípicas.

Además del análisis en las respuestas de las plantas de frijol al aluminio, se estudiaron las respuestas al cobre de plantas de frijol noduladas. Como ya se mencionó, la toxicidad por este metal afecta significativamente el crecimiento y la forma de la raíz así como la nodulación (Minnich et al. 1987). En especial nos interesó analizar si la respuesta de los nódulos de frijol en cuanto a la expresión de miRNAs ante la toxicidad por Al es común en nódulos de plantas sometidas a toxicidad por Cu o si se trata de respuestas diferentes o específicas.

Para el análisis comparativo de la toxicidad por Al o Cu en plantas de frijol noduladas, se utilizaron sistemas experimentales similares que incluyen el sistema de crecimiento hidropónico utilizado para el análisis de toxicidad por Al incluido en el tema 3.1 y reportado en Mendoza-Soto et al. (2015). En resumen, para analizar la toxicidad por Cu, se inocularon las plántulas de frijol con *Rhizobium tropici* y se crecieron en el sistema hidropónico por 10d cuando ya se observan nódulos funcionales, se procedió a la exposición a toxicidad por Cu.

Con base en trabajos anteriores sobre toxicidad por Cu (Cuypers et al. 2002, 2005), se probaron distintas concentraciones de Cu ($CuSO_4$) 50 µM, 70 µM y 100 µM), para evaluar el nivel de estrés que provoca cada una de ellas en las plantas de frijol noduladas. Se aplicó a las plantas noduladas las diferentes concentraciones elevadas de Cu, durante 7 días, ya que nos interesaba conocer las respuestas de las plantas a este estrés a largo plazo. Así, de la misma manera que para Alt, analizamos el efecto de Cu elevado en la fijación de nitrógeno de plantas con nódulos maduros. Se evaluaron parámetros fenotípicos como crecimiento radical, peso fresco y seco de raíz, y área foliar. Los resultados se muestran en las Figuras 3 y 4.

En las plantas expuestas a una concentración de 50 µM, hubo una disminución del peso fresco de la raíz (60%) (Fig 3a), una disminución en el peso seco de la raíz (54%) (Fig 3b), una disminución en la longitud de la raíz (29%) (Fig 3c) y una disminución en el área foliar (21%) (Fig 3d).

En las plantas expuestas a una concentración de 70 µM, hubo una disminución del peso fresco (50%) (Fig 3a), una disminución del peso seco de la raíz (41%) (Fig 3b), una disminución en la longitud de la raíz (9%) (Fig 3c) y una disminución del área foliar (20%) (Fig 3d).

En las plantas expuestas a una concentración de 100 µM, hubo una disminución del peso fresco de la raíz (57%) (Fig 3a), una disminución del peso seco de la raíz (54%) %) (Fig 3b), una disminución en la longitud de la raíz (14%) (Fig 3c) y una disminución del área foliar (23%)(Fig 3d).

Además las hojas de las plantas expuestas a las tres condiciones de $CuSO_4$ presentaron clorosis (Fig 4).

Se evaluó la expresión del gen marcador APX (Ascorbato peroxidasa), el cual ha sido reportado como un gen inducido por toxicidad por metales como Cobre. (Cuypers et al. 1999, Cheng-Ri et al. 2009, Smeets et al. 2009). Hubo una inducción de este gen en las plantas expuestas a las tres concentraciones, comparadas con la planta control (Fig 3e).

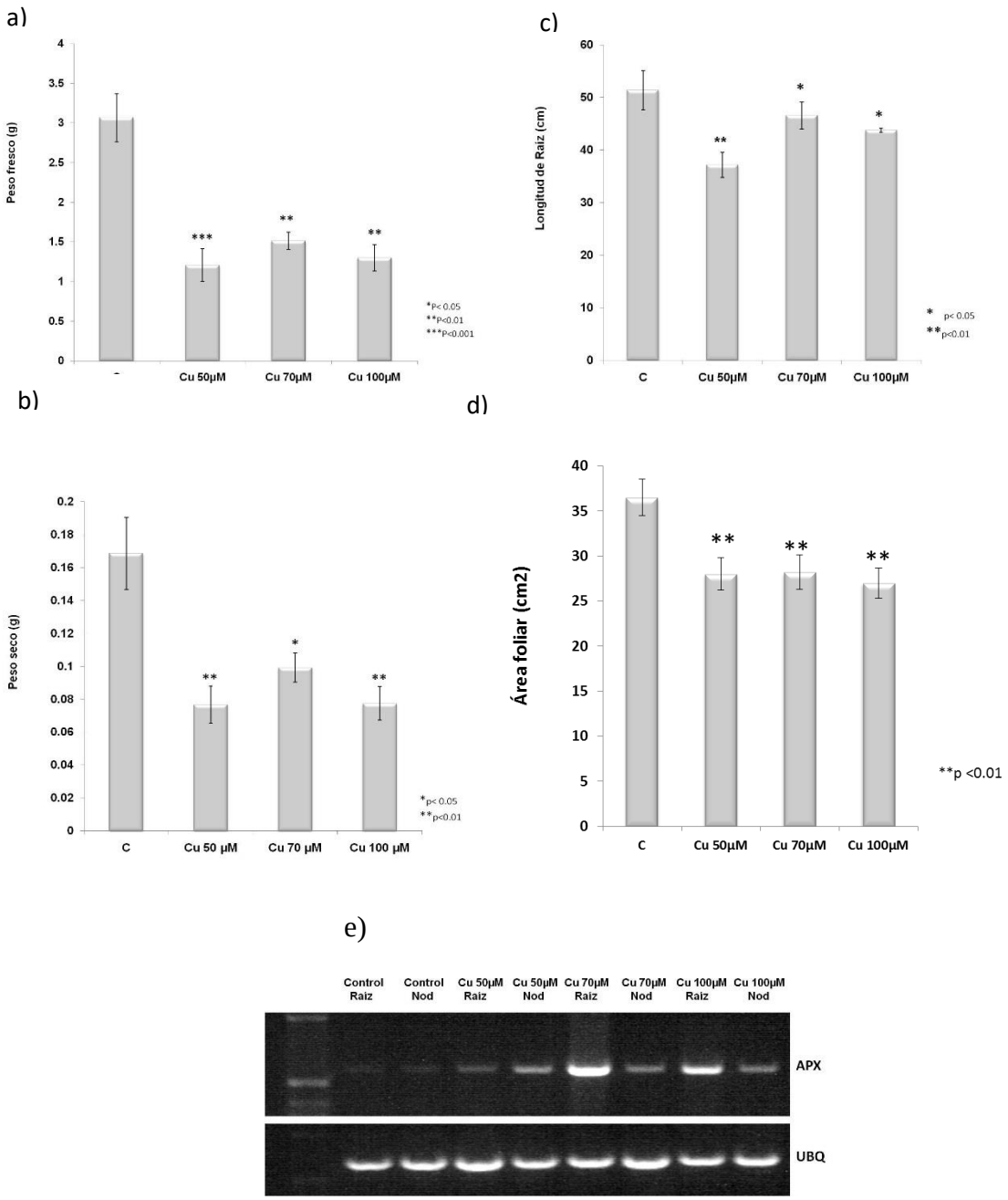

Figura 3. Medición de características fenotípicas y expresión del gen marcador APX en raíces de plantas de frijol inoculadas expuestas a diferentes concentraciones de Cobre ($CuSO_4$). a) Longitud de Raíz (cm) b) Peso fresco de raíz (g) c) Peso seco de raíz (g) n=10 d) Área foliar (cm2) e) Expresión del gen marcador APX (Ascorbato Peroxidasa). Los valores mostrados son el promedio ±ES de diez réplicas de diferentes grupos de plantas Se utilizó la prueba estadística t-Student para analizar las diferencias significativas en cada parámetro entres las plantas estresadas vs las plantas control.

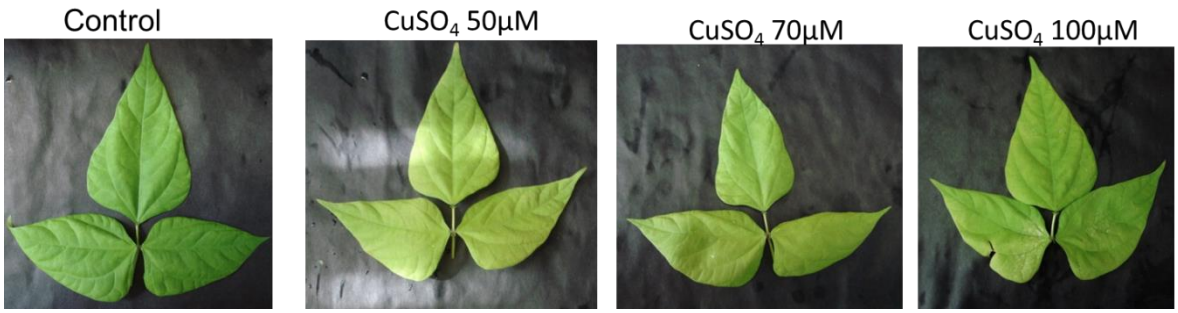

Figura 4. Clorosis presentada en hojas de plantas de frijol expuestas a distintas condiciones de CuSO4.

Se midió la actividad de la enzima nitrogenasa, se presentó una disminución de la actividad de esta enzima en las plantas expuestas a Cu 50 uM (42.5%), comparadas con las plantas Control (Figura 5), lo cual demuestra que el estrés por toxicidad por cobre afecta la fijación simbiótica de Nitrógeno.

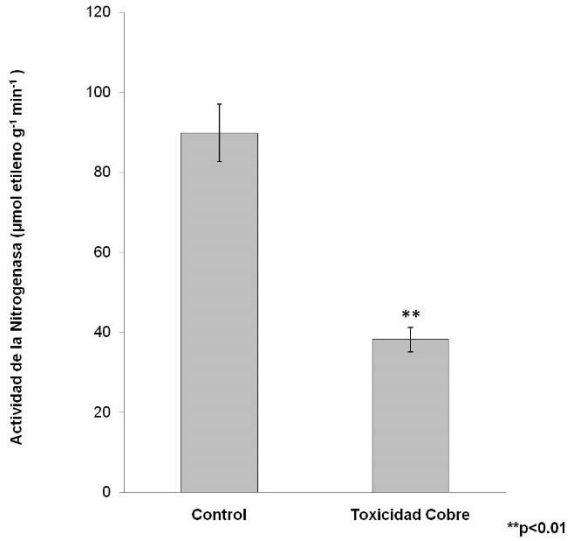

Figura 5. Actividad de la enzima Nitrogenasa en plantas de frijol noduladas expuestas a toxicidad por Cobre ($CuSO_4$ 50 µM). Los valores mostrados son el promedio ±ES de diez réplicas de diferentes grupos de plantas Se utilizó la prueba estadística t-Student para analizar las diferencias significativas en cada parámetro entres las plantas estresadas vs las plantas control.

Observando estos resultados pudimos determinar que la concentración 50 µM de CuSO4 es adecuada para los análisis de expresión de miRNAs en respuesta a toxicidad por Cobre, ya que es la concentración mínima con la cual se presentaron cambios fenotípicos de hojas y raíz, presentando una disminución en la longitud de raíz del 29% y una disminución en el área foliar del 21%. También las plantas expuestas a esta concentración mostraron un aumento en la expresión del gen marcador APX.

Considerando estos factores principales como indicadores de estrés por toxicidad de Cu se verificó si estas plantas expuestas a la concentración de 50 µM también presentaban cambios metabólicos a nivel de la actividad de la enzima nitrogenasa como un indicativo de un daño en los nódulos. Los resultados obtenidos muestran una notable disminución del 43%, confirmando así que la concentración 50 µM de CuSO4 si es la adecuada para los análisis moleculares en plantas de frijol crecidas en el sistema hidropónico.

3.2.2 Perfil de expresión de miRNAs

El perfil de expresión de miRNAs de nódulos expuestos a toxicidad por Cu se llevó a cabo con la misma metodología utilizada para el análisis de toxicidad por aluminio, incluido en el tema 3.1 y reportado en Mendoza-Soto et al. (2015). Este análisis se basa en la hibridación de miRNA-macroarrays con muestras de RNAs de nódulos, enriquecidas en RNAs pequeños, marcadas radiactivamente. El RNA se aisló de nódulos maduros de plantas de frijol expuestas durante 7 días a toxicidad por Cu (50 µM). En la tabla 1 se muestra el nivel de expresión normalizada detectada para cada uno de los miRNAs.

Tabla 1. Expresión de miRNAs en nódulos de plantas de frijol expuestas a condiciones de toxicidad por cobre en comparación con condiciones control. Los valores representan los cocientes normalizados de cada miRNA (estrés:control). Se utilizó la prueba estadística t-Student.

	Expression level	
miRNA	**Expression Ratio (± SE)**	**P-value**
miR 164	6.89 ± 0.10	0.00012
miR 396	6.22 ± 0.05	6.85 E-05
miR 170	4.75 ± 0.32	0.00040
miR 395	3.55 ± 0.13	0.01035
miR 390	2.74 ± 0.19	0.00396
miR 393	2.42 ± 0.06	0.00084
miR 166	2.39 ± 0.33	0.00378
miR 160	2.18 ± 0.18	0.01279
miR 162	1.55 ± 0.08	0.01528
miR 172	1.53 ± 0.17	0.01572
miR 168	1.29 ±0.03	0.00101
pvu-miR159.2	3.12 ± 0.27	0.00121
pvu-miR1511	2.80 ± 0.04	0.00067
pvu-miR1509	2.68 ± 0.04	0.00014
pvu-miR 2118	2.62 ± 0.03	0.00075
gma-miR1534	1.98 ± 0.03	0.00183
miR 169	-5.00 ± 0.008	5.41E-05
miR 408	-4.16 ±0.003	0.00019
miR 157	-3.57 ± 0.007	2.06 E-05
miR 398	-1.61 ±0.04	0.03510
miR 165	-1.51 ± 0.08	0.00996
miR 319	-1.44 ±0.11	0.00350
miR 156	-1.25 ± 0.04	0.02773
pvu-miR2119	-2.43 ±0.01	0.00933
gma-miR1521	-2.43 ±0.01	0.00933
mtr-miR2586	-2.00 ±0.009	0.04209

Se observó una inducción en los miRNAs conservados: miR160, miR162, miR164, miR166, miR168, miR170, miR172, miR390, miR393, miR395, miR396. Respecto a los miRNAs de frijol se observaron inducidos pvu-miR159.2, pvu-miR1509, pvu-miR1511, pvu-miR2118 y de soya el gma-miR1534 (Tabla 1). Siendo miR164, miR170, miR396, pvu-miR159.2, pvu-miR1511 y pvu-miR2118 los que presentaron los niveles de expresión más altos.

En cuanto a los miRNAs reprimidos, se detectaron los miRNAs conservados miR165, miR156, miR157, miR169, miR319, miR398 y miR408. En cuanto a los de leguminosas, de frijol se observó una represión de pvu-miR2119, de soya el gma-miR1521 y mtr-miR2586 de *Medicago truncatula* (Tabla 1). Siendo miR157, miR169, miR408, pvu-miR2119 y gma-miR1521 los que presentaron los niveles más bajos de expresión.

Para validar los resultados obtenidos de los miRNA-macroarrays se utilizaron las técnicas de Northern Blot y qRT-PCR para determinar los niveles de expresión de los miRNAs maduros. Se eligieron algunos de los miRNAs que presentaron niveles diferenciales altos, tanto de inducción como de represión.

Para el analizar los miRNAs seleccionados por medio de Northern blot, el RNA total se aisló y posteriormente fue separado por electroforesis en geles de poliacrilamida y transferido a membranas para ser hibridadas con oligonucleótidos sintéticos específicos (complementarios) para cada miRNA como sondas radiactivas. La intensidad de la señal en cada hibridación se cuantificó mediante ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA). U6 snRNA fue usado como control de carga y para la normalización de la intensidad de la señal. Se utilizó el promedio de la intensidad de señal normalizada para obtener los cocientes de expresión (estrés:control) de los miRNAs candidatos como responsivos a estrés. .

Los miRNAs elegidos para el análisis por medio de Northern Blot fueron miR164, miR170, miR396, y pvu-miR1511 los cuales presentaron una inducción en respuesta a toxicidad por Cu. Además se eligió a miR157 y miR159.2 para validar la represión encontrada en los miRNA-macroarrays.

Los resultados obtenidos en los Northern Blots se muestran en la Figura 5.

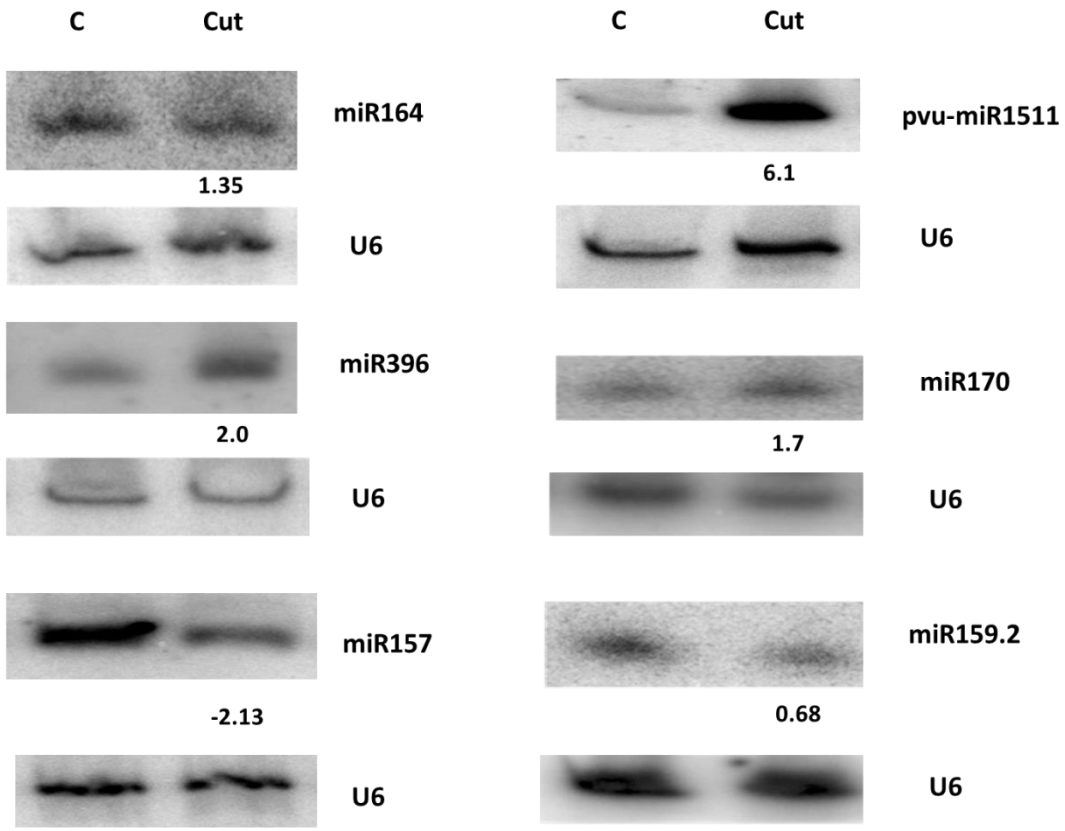

Figura 5. Análisis de miRNAs seleccionados por medio de Northern blot, de nódulos de plantas de frijol crecidas bajo condiciones control (C) o condiciones de toxicidad por cobre (Cut). La detección de snRNA U6 fue utilizado como control de carga de la muestra. El cociente de la intensidad de la señal de las bandas de hibridación de cada miRNA (estrés:control) se indica debajo de cada carril.

Observando los resultados obtenidos por medio de los análisis Northern Blot, el patrón de expresión obtenido en los miRNA-macroarrays fue confirmado para cada uno de los miRNAs analizados. Sin embargo aunque si se observó una tendencia similar en cuanto a inducción o disminución en toxicidad por cobre, hubieron variaciones en los valores del cociente de expresión obtenido en los macroarrays comparados con los obtenidos por medio del análisis Northern Blot. Esta diferencia puede ser atribuida a las diferentes sensibilidades de ambos métodos.

También utilizamos el método de qRT-PCR como otra alternativa que tiene una mayor sensibilidad para la validación de la expresión de miRNAS obtenidos en el experimento de miRNA-macroarray (Tabla 1). Para el análisis de qRT-PCR se utilizó la metodología y los oligonucleótidos específicos, ya reportados en Mendoza-Soto et al. (2015). Los miRNAs elegidos para el análisis por medio de qRT-PCR fueron miR164, miR170, miR393, miR396, pvu-miR1511, los cuales presentaron una inducción, así como miR157, miR169 y miR398 que presentaron una represión en el análisis realizado por medio de miRNA-macroarray. En la tabla 2 se pueden observar los niveles de expresión obtenidos mediante esta técnica, datos que complementan la validación de los miRNAs analizados mismos que conservan la tendencia inicialmente observada en los miRNA-macroarrays. Para estos miRNAs seleccionados se determinó también el nivel del transcrito de su gene blanco. En cada caso se observó una relación inversa, es decir, se presentó una represión del mRNA blanco cuando el miRNA se indujo, y una inducción en el mRNA blanco cuando el miRNA disminuyó (Tabla 2). La Tabla 2 muestra también los resultados anteriormente reportados de respuesta a aluminio (Mendoza-Soto et al. 2015).

Además, en este análisis se incluyó la expresión de los miRNAs y genes blanco seleccionados en nódulos de plantas de frijol expuestas a toxicidad por manganeso (Mn). Esto nos permite evaluar si las plantas de frijol expuestas a manganeso tienen

una respuesta similar a diferentes metales o es exclusiva de toxicidad a cobre o aluminio, en relación a la expresión diferencial de miRNAS. La condición de toxicidad por Mn en frijol creciendo en sistemas hidropónicos se reportó anteriormente (Valdés-López et al. 2010). Los resultados obtenidos se muestran en la Tabla 2.

Tabla 2. Expresión de miRNAs seleccionados y sus respectivos RNAm blancos en nódulos de plantas de frijol crecidas bajo condiciones control (Ctr) o condiciones de toxicidad por metales -. Toxicidad por Al (Alt), toxicidad por Cu (Cut) o toxicidad por manganeso (Mnt). Cada metal fue añadido 12 dpi durante 7 días. Cada valor representa el cociente de expresión normalizado (estrés:control) dado por el promedio de tres réplicas biológicas. Para cocientes menores de 1, se estimó el inverso del cociente y se cambió el signo. NAC1: NAM/ATAF/CUC transcription factor; SCL: SCARECROW-like protein; TIR1: transport inhibitor response 1-like protein; GRL1: growth-regulating factor 1; SP1L: SPIRAL-like protein 1; SPL: SQUAMOSA promoter-binding protein-like; HAP2: heme-activator protein transcription factor; CSD1: Cu/Zn superoxide dismutase

	Expression level	
Treatment	**miRNA**	**Target**
	miR164	NAC1
Alt	12.5	0.47
Cut	3.1	0.55
Mnt	4.7	0.54
	miR170	SCL
Alt	2.6	0.24
Cut	2.1	0.2
Mnt	4.5	0.06
	miR393	TIR
Alt	1.68	0.51
Cut	2.7	0.57
Mnt	2.62	0.43
	miR396	GRL
Alt	6.6	0.23
Cut	5.25	0.12
Mnt	5.8	0.25
	pvu-miR1511	SP1L
Alt	6.7	0.56
Cut	10	0.8
Mnt	8	0.72
	miR157	SPL
Alt	0.68	2.43
Cut	0.58	4.7
Mnt	0.45	6.3
	miR169	HAP2
Alt	0.5	1.58
Cut	0.01	2.63
Mnt	5	0.7
	miR398	CSD
Alt	0.85	1.8
Cut	0.1	4
Mnt	0.3	2.5

Respecto a la generalidad de la expresión en los nódulos de las plantas expuestas a los demás metales analizados, se observó la misma tendencia respecto al Al, en cuanto al aumento y represión en la mayoría de los casos, con excepción de miR169. Para el miR169 se observó que en el caso de toxicidad por Mn, este miRNA presentó una inducción a diferencia de la exposición a Al (resultados previamente reportados) y Cu, en los cuales se presentó una represión. Este es el único miRNA que presentó una respuesta opuesta en el caso de uno de los metales.

Aunque los demás miRNAs conservaron la tendencia entre los distintos metales, sí se observa una diferencia en cuanto a los niveles, tanto de inducción como de represión. En este sentido la diferencia más representativa se observa en el caso de miR164 que muestra el nivel más alto en Alt y en del miR398 que presenta una disminución, menos representativa en el caso de Alt en comparación de la respuesta observada en Cut y Mnt.

3.3 Caracterización de miRNAs de frijol que responden a toxicidad por metales.

3.3.1 pvu-miR1511

Tomando en cuenta los resultados observados en el estudio de toxicidad por Al (Mendoza-Soto et al. 2015), Cu y Mn, consideramos como uno de los miRNAs más interesantes para continuar su análisis, al pvu-miR1511 ya que presenta una inducción elevada en respuesta a los tres metales analizados. Lo anterior se observó utilizando las tres técnicas de cuantificación de expresión (miRNA-macroarray, Northern blot y qRT-PCR). Además este miRNA es específico de leguminosas (Arenas Huertero et al.; 2009) por lo cual podría tener una función en el proceso de fijación de nitrógeno.

Para observar la expresión específica en los nódulos se realizó la técnica de hibridación in situ para la cual los nódulos maduros de frijol fueron procesados

según Boualem et al. (2008). Se utilizaron oligonucleótidos modificados LNA (Exiqon) complementarios a pvu-miR1511 y a miR167, utilizando este último como control positivo. Para el marcaje de las sondas se utilizó la técnica de marcaje de extremos 3'OH con DIG para poder visualizar su hibridación en el microscopio.

Se observó la localización específica de pvu-miR1511 en la zona de infección del nódulo y en la zona central de haces vasculares (Fig 6) comprobando espacialmente la localización de este miRNA en nódulos maduros de frijol.

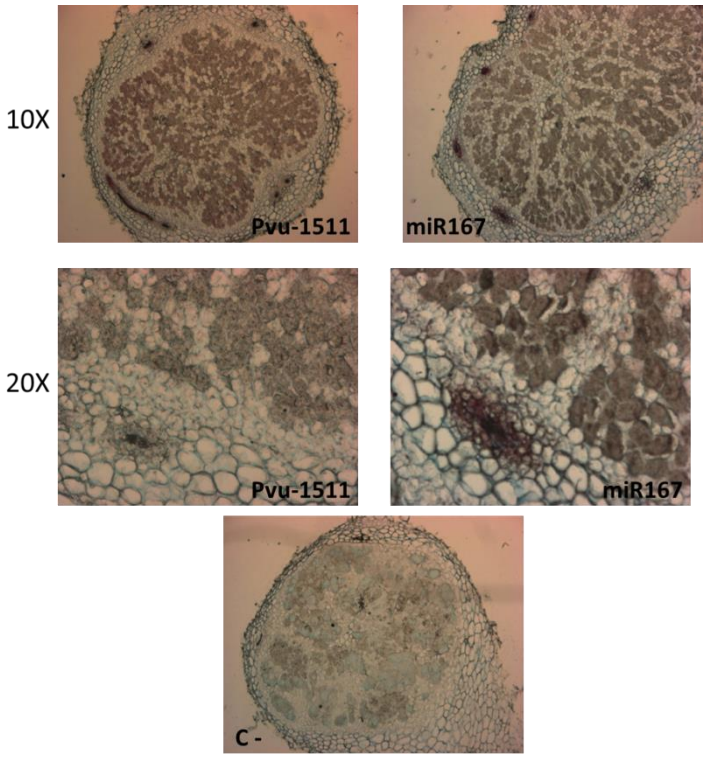

Figura 6. Expresión de pvu-miR1511 en cortes transversales de nódulos maduros de frijol. Mir167 fue utilizado como control positivo y C- es el control negativo para lo cual se utilizó la misma técnica, con el marcaje de un miRNA sintético no presente en plantas.

En trabajos anteriores se propuso al RNAm del gen 60S Ribosomal protein como el gene blanco del miR1511 de soya, lo cual se ha comprobado mediante degradoma y 5´RACE (Song et al. 2011; Luo et al. 2012), Para frijol el blanco para pvu-miR1511 que se propuso mediante análisis bioinformático fue el RNAm del gen SP1L1-related protein (Plant-specific microtubule-localized protein) (Arenas-Huertero et al. 2009).

El análisis realizado anteriormente sobre la expresión del miR1511 y SP1L1, su blanco propuesto (Tabla 2) mostró una relación inversa en cuanto a la expresión de ambos, en los nódulos de plantas expuestos a las toxicidades por metales. Para determinar si el RNAm del gen 60S Ribosomal protein también podría ser considerado como un blanco en frijol, se cuantificaron los niveles de RNAm en las plantas anteriormente analizadas buscando una correlación inversa con la expresión de pvu-miR1511. Este análisis se llevó a cabo en plantas crecidas en condiciones control así como en las diferentes toxicidades por metales anteriormente analizadas. Los datos presentados en la Figura 7 indican el cociente de expresión de las plantas estresadas respecto a las plantas control.

No se encontró correlación en cuanto a la expresión de pvu-miR1511 y el RNAm 60S Ribosomal protein en ninguna de las condiciones de toxicidad por metales en nódulos de frijol, por lo cual no se propone como un blanco para este miRNA en frijol, a diferencia de lo que ocurre en soya.

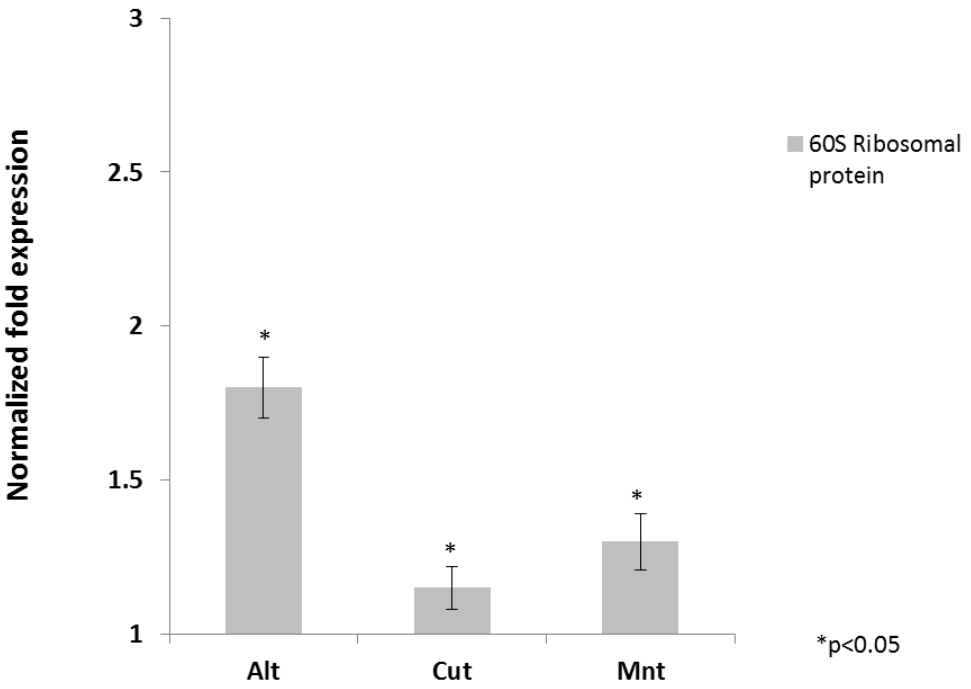

Figura 7. Expresión del RNAm correspondiente al gen 60S Ribosomal protein en nódulos de plantas de frijol crecidas bajo condiciones de toxicidad por metales. Toxicidad por Al (Alt), toxicidad por Cu (Cut) o toxicidad por manganeso (Mnt). Cada metal fue añadido 12 dpi durante 7 días. Estos valores fueron comparados respecto a la expresión de nódulos de plantas crecidas en condiciones control. Cada valor representa el cociente de expresión normalizado (estrés:control) dado por el promedio de tres réplicas biológicas.

Para determinar la función de este miRNA en los nódulos de plantas de frijol se realizó una construcción genética para su sobreexpresión utilizando el vector pTDT el cual contiene la proteína roja fluorescente como gen reportero. Se clonó la secuencia correspondiente al precursor de Gmax-miR1511 ya que la secuencia del miRNA maduro es homóloga en frijol, río abajo del promotor constitutivo y fuerte 35SCaMV (Fig. 8).

Con esta construcción genética se generaron plantas compuestas, con el sistema radical transformado y el aéreo no transformado (Estrada-Navarrete et al. 2007). Para la transformación de las plantas, se utilizó la cepa *Agrobacterium rhizogenes* K599 que contenía el vector vacío o el pTDT OE-1511 (Fig. 8). Las plantas compuestas seleccionadas fueron crecidas bajo condiciones de crecimiento control en macetas con vermiculita y regadas con solución nutritiva B&D (Broughton and Dilworth; 1971).

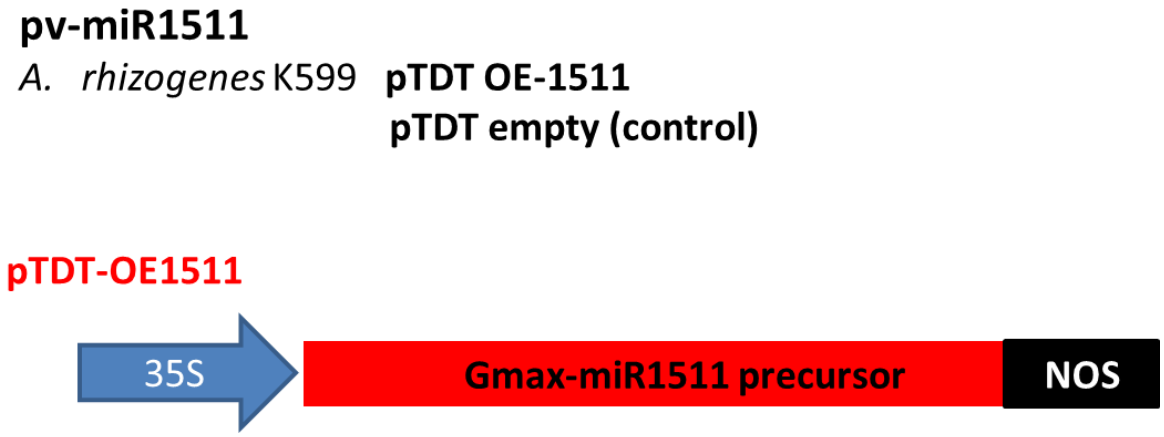

Figura 8. Construcción genética para la sobreexpresión de Gma-miR1511.

Se cuantificaron los niveles de expresión de pvu-miR1511 y del RNAm blanco SP1L1, en ambos grupos de plantas transformadas, es decir, en las que contenían el vector vacío y en las que contenían la construcción para la sobreexpresión (pTDT OE-1511). Este análisis se realizó en nódulos y raíces mediante la técnica de qRT-PCR.

Las plantas transformadas con la cepa con pTDT OE-1511 mostraron niveles altos en comparación con las plantas transformadas con la cepa con el vector vacío. Este

patrón se observó en ambos órganos, tanto en raíces como en nódulos, confirmando la sobreexpresión del miRNA (Fig. 9).

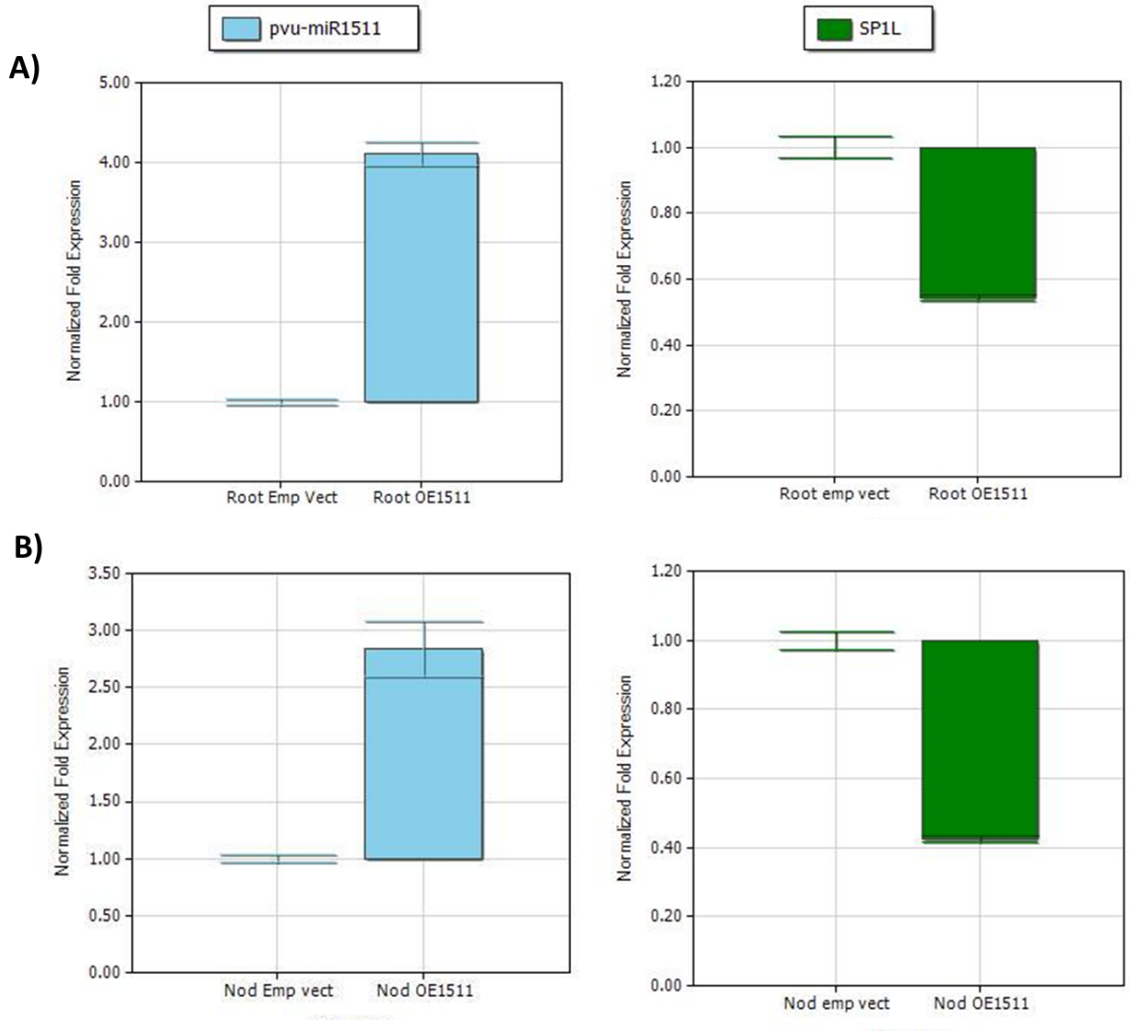

Figura 9. Expresión de pvu-miR1511 y del RNAm de SP1L en raíces y nódulos de plantas de frijol transformadas crecidas en condiciones normales. Cada valor representa el cociente de expresión normalizado (OE1511: VECTOR VACIO) dado por el promedio de diez réplicas biológicas.

Además, estas plantas presentaron en los niveles de expresión de SP1L, una relación inversa respecto a los niveles del miRNA (Fig. 9), por lo cual éste es un candidato importante para ser el blanco de este miRNA.

Para determinar alguna función de este pvu-miR1511, el cual se ha visto inducido ante condiciones de estrés por toxicidad de metales (Tabla 2), se analizó el fenotipo de este órgano en las plantas sobreexpresantes comparadas con las plantas control (vector vacío).

El tamaño de los nódulos se observó visiblemente distinto, mostrando un menor tamaño los nódulos de las plantas sobreexpresantes, por lo tanto también presentaron un menor peso seco. En cuanto a la coloración, los nódulos de las plantas control mostraron un color más blanco en comparación con el tono rosado presente en las sobreexpresantes (Fig. 10).

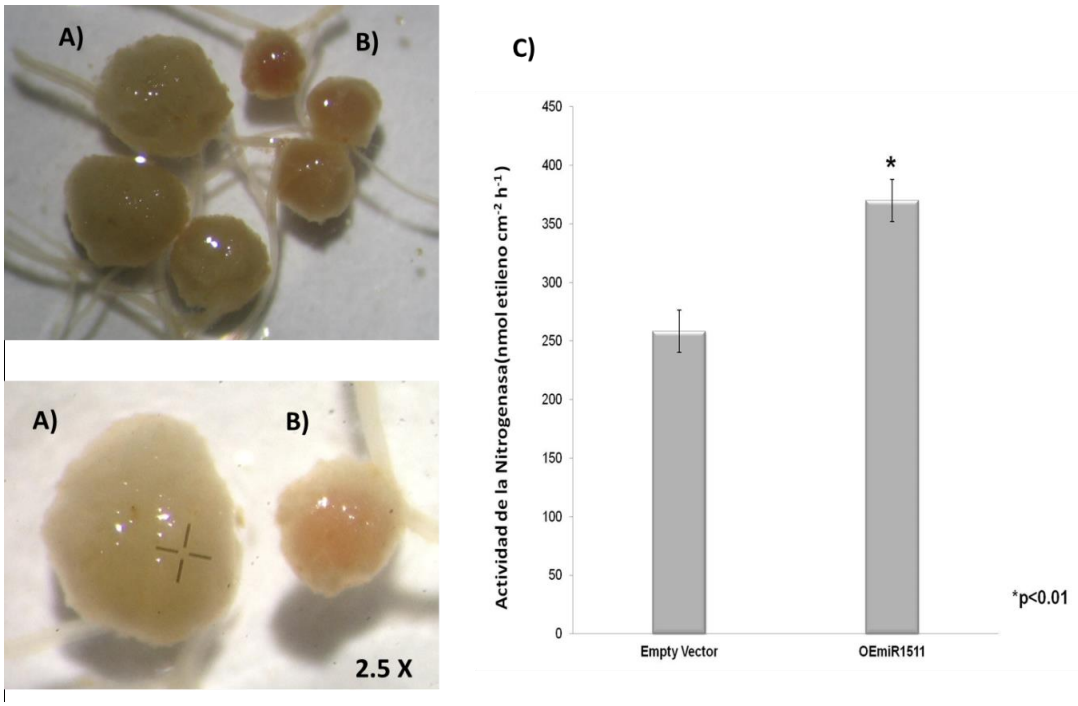

Figura 10. Fenotipo de nódulos de plantas sobreexpresantes y control. A) Nódulos de plantas control las cuales contienen el vector vacío. B) Nódulos de plantas sobreexpresantes, las cuales contienen vector con OE 1511. C) Actividad de la enzima nitrogenasa. n=10

Buscando una correlación del color presentado en las plantas OE1511 con la funcionalidad de los nódulos, se midió la actividad de la enzima nitrogenasa. Los nódulos eficientes son rosas debido a la presencia de la leghemoglobina, la cual controla el flujo de oxígeno a la bacteria (Lambers et al. 2008). Las plantas sobreexpresantes presentaron una mayor actividad de la enzima nitrogenasa (Fig. 10), por lo cual son más eficientes comparados con los nódulos de las plantas control.

De acuerdo a los resultados obtenidos, pvu-miR1511 causa efectos en los nódulos de frijol, tanto en tamaño como en funcionalidad por lo que este miRNA podría tener un papel importante en este órgano.

3.3.2. MiR398

Se participó en la caracterización de este miR398 en plantas de frijol, específicamente en la respuesta ante toxicidad y deficiencia de cobre. En este trabajo Naya et al. (2014), validó al RNAm de Nod19 como un gen blanco nuevo en el cual ejerce su acción miR398. Mediante el análisis de expresión de miR398b y sus genes blanco CSD1 y Nod19, en raíces, nódulos y hojas de frijol, se comprobó el importante papel que tiene este miRNA en la homeostasis de Cu. En las plantas expuestas a toxicidad por Cu, la expresión de miR398b disminuye y Nod19 y CSD1, que participa en la detoxificación de especies reactivas de oxígeno (ROS), presentaron un aumento en su expresión. En las plantas expuestas a deficiencia de cobre, se observó una regulación opuesta, es decir, un aumento en miR398b y un nivel más bajo de CSD1 lo cual permite utilizar el escaso cobre disponible en proteínas esenciales que contienen cobre. Estos resultados se pueden observar en el anexo.

4. Discusión

La fijación simbiótica de nitrógeno y la producción del cultivo de frijol son afectadas por la toxicidad por metales como Al y Cu, siendo el primero el que más afecta a esta leguminosa ya que se encuentra en altas cantidades en los suelos ácidos, en los cuales crece el frijol (Graham et al. 2003). En este trabajo analizamos la respuesta de raíces y nódulos maduros de plantas de frijol inoculadas con rhizobia y expuestas a toxicidad por Al, así como la respuesta de las raíces en plantas fertilizadas con nitrógeno

El principal efecto observado en las plantas de frijol expuestas a Alt fue una disminución en la longitud de raíz. Este efecto es observado aún a tiempos cortos de exposición al estrés, encontrando una disminución de 2 cm de longitud en la raíz principal (Mendoza-Soto et al., 2015 Fig 1). Este resultado coincide con estudios previos en frijol en los cuales se ha señalado que la zona de transición y elongación de la raíz son los blancos principales de Alt lo cual promueve una rápida inhibición de la elongación de la raíz (Rangel et al., 2007; Yang et al., 2009). Después de un periodo largo (7 días) de exposición a Alt se observó una disminución notable en la longitud de raíz tanto en las raíces inoculadas como en las fertilizadas, siendo más evidente en las plantas inoculadas, lo cual confirma estudios previos en los cuales se propone que las leguminosas noduladas son más sensibles a toxicidad por metales como Al y Mn, que las plantas fertilizadas con N (Hungria y Vargas, 2000).

Los nódulos de las plantas de frijol expuestas a Alt mostraron una actividad de la nitrogenasa reducida, en el caso de las expuestas a 7 días disminuyó un 50%, mientras que en las expuestas a 24 hrs disminuyó un 40% (Mendoza-Soto et al. 2015; Fig .2), esto indica el daño directo del exceso de Alt en el proceso de la fijación simbiótica de nitrógeno, acumulación que fue evidente en la zona de infección de nódulos maduros (Mendoza-Soto et al., 2015 Fig. 3). Generalmente las bacterias bajo un exceso de Al utilizan los sistemas de transporte de Fe para la toma de Al, lo

cual interfiere con la captura de Fe, el cual es un micronutriente esencial requerido para la actividad de la nitrogenasa en rhizobia. (Davis et al. 1971, Rogers et al. 2001). Especies como *Sinorhizobium meliloti* y *Bradyrhizobium* creciendo en condiciones ex planta son extremadamente sensibles al Al debido a los efectos en las actividades enzimáticas para la reducción de nitrato y nitrito, nitrogenasa y toma de hidrogenasa (Arora et al., 2010). Además una alta concentración de Al en un medio de cultivo ácido, precipita este elemento como $Al_2(PO_4)_3$ lo cual reduce la disponibilidad de fósforo. Una disminución en la concentración de fósforo se ha asociado con una actividad disminuida de la nitrogenasa (Schulze et al. 2011).

Las raíces inoculadas estresadas mostraron síntomas como acumulación de Al en el ápice radical, así como acumulación de ROS, callosa y lipoperoxidación (Mendoza et al. 2015; Fig 3). La acumulación de la callosa y H_2O_2 (ROS) fue colocalizada en la zona de elongación de la raíz similar a lo observado en maíz (Jones et al., 2006). Esta co-localización coincide con la explosión de ROS con un incremento en el Ca^{2+} citoplasmático, lo cual promueve la activación de la enzima callosa sintetasa (Jones et al., 2006)

Las plantas expuestas a Alt inducen la acumulación de callosa y promueven que la membrana y la pared celular se vuelvan más rígidas; proponemos que esta rigidez podría funcionar como una barrera protectora contra la toma continua del Al en nódulos y raíces.

En el caso de las plantas expuestas a Cobre, también presentaron una disminución en la longitud de raíz lo cual ha sido observado en estudios anteriores en frijol y otras plantas (Cuypers et al. 2002; Marschner, 2011; Sheldon A. y Menzies NW; 2004). Una de las diferencias evidentes comparadas con las plantas expuestas a Alt, fue la disminución del área foliar (Fig. 3), además de la presencia de clorosis (Fig. 4). Este fenómeno se ha relacionado con el efecto directo que tiene los iones de Cu en la fotosíntesis, en específico en la estructura de las membranas de los

tilacoides debido a la peroxidación y estrés oxidativo, dando como resultado una ineficiencia en la fotosíntesis, ya que este cambio de estructura en los cloroplastos lleva a un efecto en el transporte de electrones así como la pérdida en la actividad fotoquímica en los fotosistemas I y II (Caspi et al. 1999; Mysliwa-Kurdziel et al. 2004). Por otro lado el Cu también inhibe la acumulación de la clorofila, removiendo el Mg de la misma de ambos complejos antena y centros de reacción, dañando así la estructura y función de la clorofila (Küpper et al. 2003). En *Brassica pekinensis* se ha reportado una reducción considerable del contenido de clorofila después de 6 días de exposición a toxicidad por Cu (Xiong et al. 2006).

Los nódulos de plantas expuestas a Cut también presentaron una fijación de nitrógeno disminuida de más de un 50% (Fig. 5). En soya, se ha atribuido este efecto en la actividad de la nitrogenasa a la alteración de la síntesis de la leghemoglobina y una proliferación limitada de bacteroides.

La disminución en la fijación de nitrógeno y de la leghemoglobina es debido a una disminución de Fe, tal como sucede en el caso del Al, como resultado de una competencia (Younis, 2007). Este efecto no se observa en respuesta a todos los metales ya que se ha observado que el cadmio (Cd) aplicado a una concentración de 100 µM no causa efecto en la actividad de la nitrogenasa, lo cual se atribuye a una resistencia en la cepa *Rhizobium leguminosarum biovar viciae* ante toxicidad por metales como Cd (El-Enany y Abd-Alla; 1995).

En cuanto a la identificación de miRNAs responsivos a metales, este es un primer paso para desentrañar su papel como reguladores globales relevantes para la adaptación de los tejidos de plantas y defensa a este estrés abiótico. Identificamos 28 miRNAs que responden a Alt y 26 que responden a Cut en nódulos maduros de frijol (Tabla 1).

Algunos de los miRNAS responsivos fueron validados por medio de las técnicas de Northern Blot y qRT-PCR. Esta última incluyó el análisis de la expresión de ocho

miRNAs en nódulos expuestos a toxicidad por Mnt, además de los nódulos expuestos a Cut y Alt (Tabla 2). La expresión de los miRNAs predichos mostró la tendencia esperada basada en los resultados obtenidos en el miRNA-macroarray; esta tendencia se observó en los análisis con ambas técnicas (Fig. 5; Tabla 2) aunque los cocientes mostraron variaciones, esto fue debido a la diferente sensibilidad de cada método utilizado para la validación. Además la expresión de los RNAm blanco predichos mostró la relación inversa esperada entre el miRNA y el blanco (Tabla 2).

Los resultados mostraron una respuesta similar en los miRNAS y sus RNAm blanco en los tres tipos de estrés por diferentes metales, con excepción de miR169 que mostró una inducción en los nódulos de plantas expuestos a Mnt comparado con la represión observada en Alt y Cut.

El miRNA que mostró mayor inducción en los nódulos de frijol sometidos a Alt, Cut y Mnt fue el miR164. Este miRNA y su correspondiente blanco, el RNAm NAC1, se han evaluado en *Medicago truncatula* mostrando una alta expresión de NAC1 en los nódulos simbióticos y la sobreexpresión de miR164 conduce a una reducción en el número de nódulos (D'Haeseleer et al. 2011). Sin embargo, este miRNA se reportó como disminuido en raíces de soya estresadas por Al y en plántulas de Medicago estresadas por Hg (Zeng et al. 2012, Zhou et al. 2012), lo que indica la posibilidad de un papel diferente de este miRNA y su factor de transcripción blanco NAC1 en la respuesta de los nódulos de frijol a la toxicidad por metales. En Arabidopsis, NAC1 suprime la señalización de auxinas, afectando el crecimiento de la raíz (Guo et al., 2005). Las auxinas han sido implicadas en la mediación de señales de nitrógeno; sus niveles en las raíces se regula dependiendo del estatus en cuanto a la disponibilidad o contenido de nitrógeno de la planta (Kiba et al. 2011). La disminución de la actividad de la nitrogenasa detectado en plantas de frijol noduladas estresadas por Alt y Cut (Mendoza-Soto et al. 2015 Fig 1; Fig. 5)

conduciría a un bajo contenido de N, por lo que proponemos que la regulación de NAC1 mediada por miR164 puede modificar la señalización de auxinas en relación con el estado reducido de nitrógeno en nódulos de frijol estresados.

El miR396 mostró una inducción en su expresión y su blanco, el factor de transcripción GRF, una disminución en los nódulos de frijol expuestos a Cut, Alt y Mnt. GRF está involucrado en el control de la proliferación celular durante el desarrollo de hojas (Rodriguez et al. 2010). Este miRNA conservado, también es inducido en raíces de soya (Zeng et al. 2012) pero reprimido en ápices radicales de *M. truncatula* (Chen et al. 2012) bajo toxicidad por Al. La regulación contrastante de miR396/GRF en estas leguminosas podría estar relacionada con los diferentes tipos de nódulos. *M. truncatula* forma nódulos indeterminados los cuales tienen un meristemo persistente y su meristemo apical produce continuamente nuevas células, quizás con la participación de GRF, mientras que en soya y en frijol se forman nódulos determinados. En los nódulos de frijol estresados por metales, GRF podría participar en el recambio de células dañadas por el estrés.

En los nódulos de frijol estresados por metales, miR398 y miR169, mostraron una disminución en su expresión mientras que sus respectivos blancos, la enzima CSD y el factor de transcripción HAP2, fueron inducidos (Tabla 2). La acumulación de ROS (H_2O_2) y por lo tanto un estado de estrés oxidativo fue evidente en los nódulos de frijol expuestos a Alt (Mendoza-Soto et al. 2015, Fig. 3).

La enzima CSD cataliza la dismutación del superóxido en peróxido de hidrógeno; el aumento de la concentración y por lo tanto de la actividad de esta enzima, provocado por la represión de miR398, es de suma importancia para aliviar la acumulación de ROS tóxicas como un mecanismo de defensa primario ante el estrés por toxicidad de metales (Sunkar et al. 2007, Mendoza-Soto et al. 2012).

El miR169 responde tanto a estreses abióticos, tales como la sequía y la salinidad (Zhao et al., 2007, 2009), y al estrés biótico, como *P. syringae pv. tomato* en Arabidopsis (Zhang et al., 2011) y *Fusarium virguliforme* en soya (Radwan et al. 2011). Es posible que este miRNA también responda a una alta acumulación de ROS generada por varios tipos de estrés incluyendo la toxicidad de metales.

En este trabajo, caracterizamos la respuesta de nódulos de frijol ante estrés por Alt e identificamos miRNAs responsivos a Alt y a otros metales como Cut y Mnt, comparamos su expresión relativa con la de sus RNAm blanco; los cuales podrían tener un papel importante en el mantenimiento o función de los nódulos bajo estrés por metales.

Un miRNA candidato importante es el pvu-miR1511 el cual es específico de leguminosas (Song et al. 2011, Peláez et al. 2012), el cual en soya no presenta cambios en su expresión en raíces estresadas por Alt pero si presentó una inducción en nódulos de frijol expuestos a toxicidad por metales (Tabla 2), por lo cual se analizó más profundamente haciendo uso de genética reversa por medio de la generación plantas compuestas.

Determinamos que el posible blanco de este miRNA es SP1L, ya que además de encontrar una relación inversa en los niveles de éste respecto a pvu-miR1511 en los nódulos de las plantas expuestas a distintas toxicidades de metales, también se observó el mismo comportamiento en cuanto a esta relación en la expresión, en las plantas transformadas para la sobreexpresión de este miRNA.

En Arabidopsis se ha estudiado la familia de genes SPIRAL (SPR), a la cual pertenece SP1L, se les ha atribuido la función del control direccional de la elongación celular (Furutani et al. 2000). Gracias a la caracterización de mutantes de genes de esta familia se ha relacionado que este control de la elongación celular se lleva a cabo mediante procesos dependientes de microtúbulos, controlando de

esta manera la elongación y expansión celular anisotrópicamente (Furutani et al. 2000; Nakajima et al. 2004).

Las plantas sobreexpresantes de SPR1 presentan un aumento en la elongación celular por lo cual esta proteína se considera como uno de los elementos importantes en la regulación celular (Nakajima et al. 2004).

Las auxinas son hormonas que se han relacionado con la estimulación de la elongación celular y aunque no se han descrito los detalles moleculares de cómo los genes SPR1 actúan en esta regulación celular, las auxinas podrían tener un papel importante en este proceso. La actividad de la enzima nitrogenasa en las plantas sobreexpresantes de pvu-miR1511 presentaron un aumento. Como se ha comentado anteriormente, las auxinas han sido implicadas en la mediación de señales de nitrógeno (Kiba et al. 2011); si hubiese una alteración en los niveles de auxinas, aún sin la disminución de la actividad de la nitrogenasa provocada por algún estrés, esta alteración podría estar enviando una señal para un aumento en la actividad de la nitrogenasa en los nódulos de las plantas sobreexpresantes de pvu-miR1511 (Fig. 10). Para determinar si esto ocurriese se tendrían que medir los niveles de auxinas en estas plantas de frijol que sobreexpresan miR1511 y compararlos con las plantas control; de esta forma se podría analizar si las auxinas pudieran tener una relación con la regulación de este miRNA.

La diferencia en el tamaño de los nódulos (Fig. 10) podría deberse también a una alteración en los niveles de esta hormona.

Si bien hay algunos estudios que comprueban la función en cuanto a la regulación de la expansión y elongación celular de algunos de los genes de esta familia de genes SPR, aún hay varias proteínas de este grupo que tienen función desconocida por lo cual SP1L podría ser un candidato para profundizar más sobre su función en los nódulos de frijol y en específico en la respuesta a toxicidad por metales.

Los datos aquí presentados brindan una base para análisis posteriores que conduzcan a la demostración de las funciones específicas de los miRNAs candidatos de nódulos de frijol utilizando enfoques genéticos o biotecnológicos.

5. Conclusiones

- Las plantas de frijol noduladas expuestas a toxicidad por aluminio presentan alteraciones fisiológicas en comparación con las plantas control. Entre las alteraciones observadas destacan la lipoperoxidación, acumulación de ROS, acumulación de callosa y muerte celular, en raíces y nódulos de esta leguminosa.
- Mir164, miR170, miR393, miR396, pvu-miR1511 presentan un aumento en su expresión en los nódulos de plantas de frijol expuestas a toxicidad por los metales Al, Cu y Mn.
- Mir157 y miR398 presentan una disminución en su expresión en los nódulos de plantas de frijol expuestas a toxicidad por los metales Al, Cu y Mn.
- Las plantas sobreexpresantes de pvu-miR1511 presentaron una disminución del RNAm del gen SP1L1, lo cual sugiere que este sería el blanco para este miRNA en las raíces y nódulos de plantas de frijol.
- Las plantas sobreexpresantes de pvu-miR1511 mostraron una disminución en la actividad de la nitrogenasa, así como una disminución en el tamaño de los nódulos, sugiriendo que este miRNA tiene un papel importante en la función de este órgano en plantas de frijol.

6. Perspectivas

- Análisis de plantas silenciadas para el miRNA pvu-miR1511 por medio de la técnica “mimicry” (Franzo-Zorrilla et al. 2007) en la cual se inserta una secuencia capaz de secuestrar a pvu-miR1511 maduro, siendo éste un

mecanismo para inhibir la actividad del miRNA, obteniendo como resultado un “silenciamiento” del miRNA. Este análisis se realizará a nivel de fenotipo y de expresión.

- Comprobación que el RNAm del gen SP1L1 es el blanco de pvu-miR1511, por medio de la validación mediante el ensayo 5´RACE, el cual nos permite identificar el sitio de corte realizado por el miRNA.
- Análisis microscópico de las raíces y los nódulos de las plantas transformadas para determinar si existe un fenotipo característico de estas plantas y así poder determinar la función de pvu-miR1511.
- Análisis de la expresión y el fenotipo de plantas transformadas con OEprec-pvu-miR1511 expuestas a toxicidad por Al y Cu.

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Figura 1. Pearson Publications, disponible en:
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ANEXO 8.1

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PLOS ONE

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

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Abstract

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

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Introduction

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

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

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

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

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

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

Results and Discussion

MiR398 isoforms and target genes

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

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

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

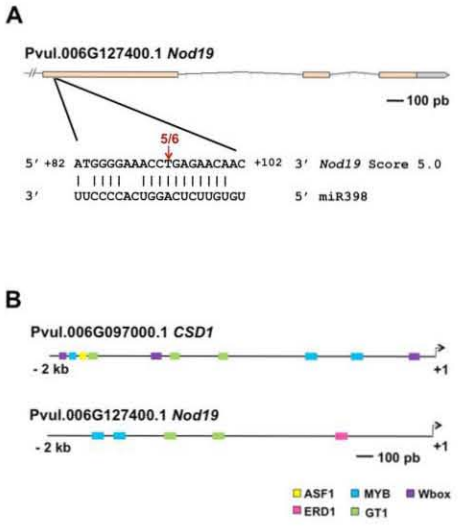

Figure 1. Common bean target genes for miR398. (**A**) *Nod19* gene structure according to the *P. vulgaris* genome sequence V.1.0 (www.phytozome.net). Exon regions are indicated with salmon-colored boxes and introns with black lines. The experimental validation of miR398 cleavage site was performed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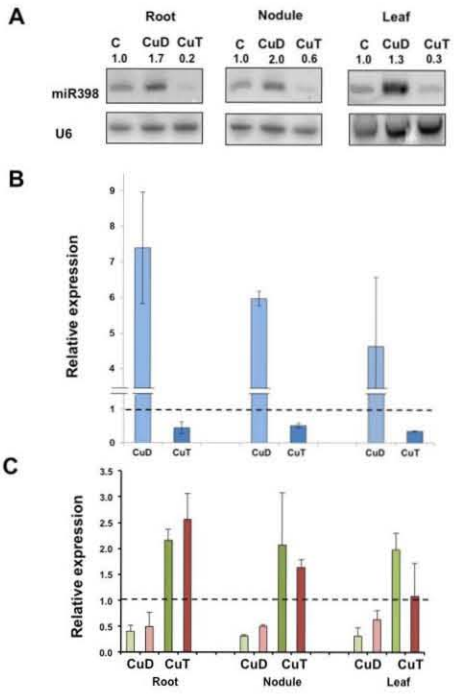

Figure 2. Expression pattern of miR398 and target genes *CSD1* and *Nod19* in tissues from common bean plants under copper deficiency (CuD) or copper toxicity (CuT). **(A)** miR398 levels in roots, nodules and leaves of plants grown under control (C) or stress (CuD or CuT) conditions were detected by Northern blot analysis using U6snRNA as loading control. Signal intensity of the hybridization bands was calculated and the expression ratio (stress:control) was obtained. Relative expression of **(B)** miR398b (blue) and of **(C)** target genes CSD1 (green) and Nod19 (red) in roots, nodules and leaves of plants grown under CuD (light colors) or CuT (dark colors) as determined by qRT-PCR. Values were normalized to the value from the C condition that was set to 1 as indicated with a dashed line. Values represent the average ± SD from three biological replicates.
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reported for other (monocot and dicot) plants such as Arabidopsis, rice, tomato, pea and *Phaseolus acutifolius* [35]. In addition to the regulation of *MtN19* during nodulation, it is induced in response to stress such as high light or drought stresses in Arabidopsis [36] and treatment with Bruchin B, a lipid-derived insect elicitor, in pea pods [35]. On this basis, it has been proposed that MtN19-like proteins that belong to the Stress Up-Regulated Nodulin 19 (SURNod19) family (Protein Families Database of Alignments and HMMS, pfam.sanger.ac.uk/) play important roles in plant stress responses [35]. To our knowledge *MtN19*-like transcripts have only been proposed as miR398 target in soybean [10], but not in *M. truncatula* or other plants. In this work we present evidence of the miR398b-mediated regulation of *Nod19*, together with *CSD1*, in common bean plants under abiotic and biotic stress conditions.

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

Response of miR398b and its target genes to copper stresses

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

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

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

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

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

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

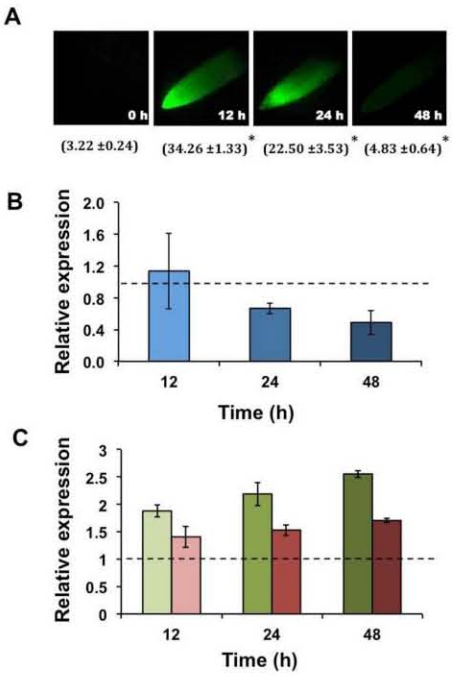

Figure 3. Reactive oxygen species (ROS) content and expression pattern of miR398 and target genes *CSD1* and *Nod19* in roots exposed to high Cu (CuT). Measurements were done at initial time (0 h) and after 12, 24 and 48 h of high Cu (70 μM $CuSO_4$) application. (A) Histological (fluorescence) detection of ROS accumulation in CuT stressed root tips using 2′,7′- dichlorodihydrofluorescein diacetate (H_2DCF-DA). The values in parenthesis indicate the average integrated fluorescence intensity per unit area of root tissue ±SD. Asterisk: Student's *t* test, $P \leq 0.05$. Relative expression, determined by qRT-PCR, of (B) miR398b (blue) and of (C) target genes *CSD1* (green) and *Nod19* (red) in CuT-stressed roots at the indicated time points. Values were normalized to the value from the C condition that was set to 1 as indicated with a dashed line. Values represent the average ± SD from three biological replicates.
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CSDs are dispensable for ROS detoxification since they can be replaced by FSDs, PC is essential for the photosynthetic electron flow in higher plants [26]. In Arabidopsis miR398 increases in Cu-starvation and it is involved in the mechanism to regulate Cu-containing proteins [26,54]; our data indicate that a similar mechanism holds for Cu-deprived common bean plants. The levels of miR398 highly increased in roots, nodules and leaves of CuD bean plants lead to very low level of *CSD1* (Fig. 2) that would prioritize the delivery of limited Cu to PC.

Over-expression of miR398 in composite plants

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

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

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

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

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

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

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

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

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

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

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

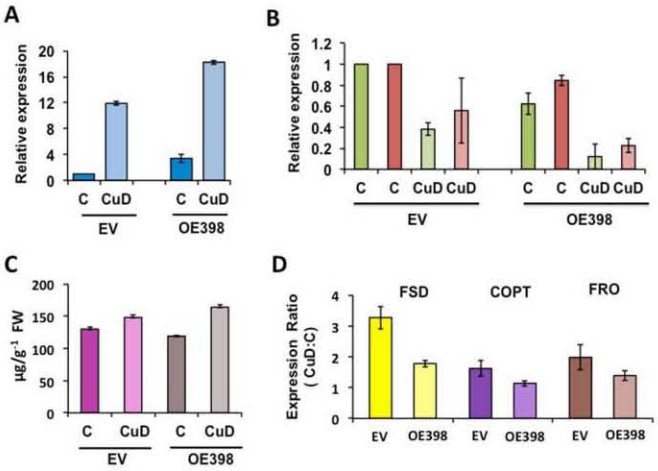

Figure 4. Effect of miR398b over-expression in transgenic roots from composite plants grown under CuD. Composite plants were obtained through *A. rhizogenes* transformation with EV or with OE398 plasmid, these were grown in control (sufficient nutrient) condition (C) or in CuD stress condition. (**A**)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

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

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

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

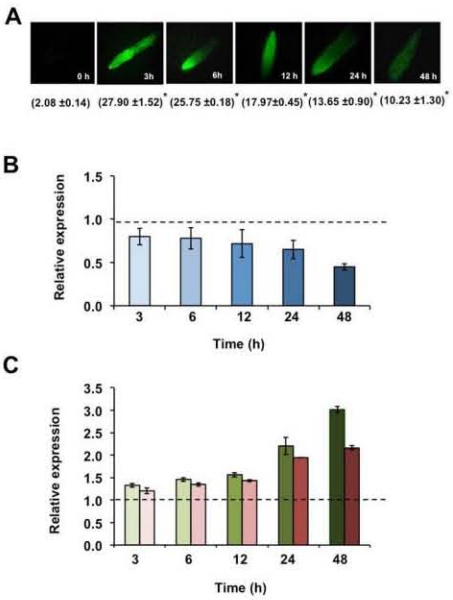

Figure 5. ROS content and expression pattern of miR398 and target genes *CSD1* and *Nod19* in roots inoculated with *Rhizobium tropici*. Measurements were done at initial time (0 h) and 3, 6, 12, 24 and 48 h after inoculation with *R. tropici*. (A) Histological (fluorescence) detection of ROS accumulation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

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

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

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

Conclusions

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

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

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

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

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

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

Materials and Methods

Plant material and growth conditions

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

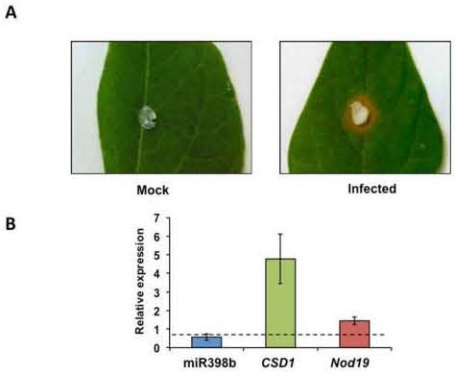

Figure 6. Expression pattern of miR398b and target genes *CSD1* and *Nod19* in common bean leaves infected with *Sclerotinia sclerotiorum*. (A) Mock (left) or *S. sclerotiorum* infected (right) common bean leaves after 24 h. (B) Relative expression of miR398b (blue) and of target genes *CSD1* (green) and *Nod19* (red) determined by qRT-PCR; values were normalized to the value from mock that was set to 1 as indicated with a dashed line.
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7d-old plants were inoculated with 10 mL of saturated liquid culture of *Rhizobium tropici* CIAT899. After 14d post inoculation when bean plants have developed small nodules actively fixing atmospheric N_2, stress was applied. For Cu toxicity (CuT) the nutrient solution was supplemented with 70 µM $CuSO_4$ and for Cu deficiency (CuD) the nutrient solution was deprived of Cu, for control condition the nutrient solution with 1 µM CuSO4 was maintained. Under the Cu-stress conditions used plants presented characteristic visual symptoms. In each CuT experiment the expression of *APX* (Phvul.011G071300), marker gene for CuT [48], was determined by qRT-PCR (see below). In each CuD experiment, the expression of *FSD* (Phvul.007G135400.1), marker gene for CuD [41], was determined. Increased expression of the marker genes indicated the stress-nature of the treatment used (data not shown). Roots, mature nodules or leaves from inoculated plants were harvested at 7d post stress. For analysis of roots at early stages of rhizobia infection, plants were inoculated as described and roots were collected at 0h, 3h, 6 h, 12 h, 24 h and 48 h post inoculation. For non-symbiotic conditions plants were grown in full-nutrient solution and the same $CuSO_4$ concentration was used for CuT condition, roots were collected at 12 h, 24 h and 48 h.

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

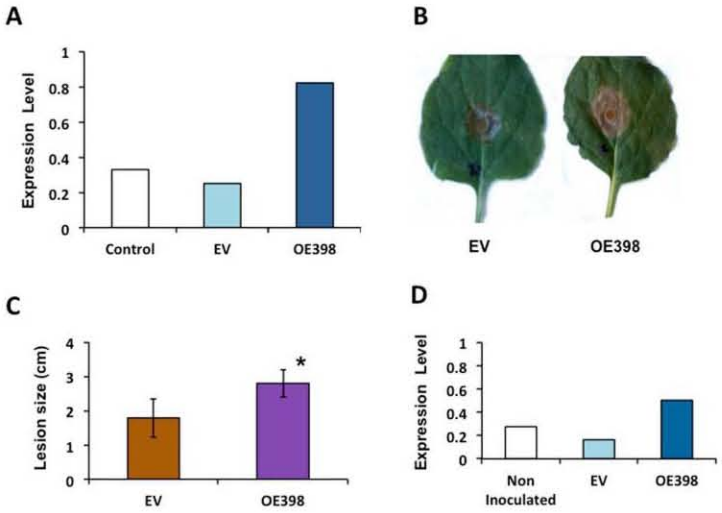

Figure 7. Effect of miR398b transient over-expression in *Nicotiana benthamiana* leaves infected with *Sclerotinia sclerotiorum*. *N. benthamiana* leaves were infiltrated with water (Control) or with *A. tumefaciens* bearing EV or OE398 plasmids and miR398b expression level was determined 3d after infiltration **(A)**. Subsequently, infiltrated leaves (EV or OE398) were inoculated with *S. sclerotiorum*. Characteristic fungal lesions **(B)** quantified by measuring the infection halo; asterisk: Student's *t* test, $P \leq 0.01$ **(C)** and miR398b expression levels determined by qRT-PCR **(D)** at 48 h after fungal infection.
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described before [75]. Transgenic roots were harvested for gene expression analysis through qRT-PCR.

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

ROS detection

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

DNA sequence analysis of cis-elements

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

RNA isolation

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

Target validation by 5′RACE

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

RNA gel blot analysis

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

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

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

Plasmid construction

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

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

Fungal infection assay

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

Plant transformation

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

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

Supporting Information

Table S1 Expression profile of miR398a.
(DOC)

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

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Author Contributions

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

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