UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

CENTRO DE CIENCIAS GENÓMICAS

DOCTORADO EN CIENCIAS BIOMÉDICAS

REGULACIÓN TRANSCRIPCIONAL Y POST-TRANSCRIPCIONAL DE LAS RESPUESTAS DE FRIJOL A ESTRÉS NUTRICIONAL

T E S I S

QUE PARA OBTENER EL GRADO DE

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PRESENTA

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Oswaldo Valdés López

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CONTENIDO

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VII. 7 Material y Métodos para el análisis de las respuestas de frijol a la toxicidad por Mn^{+2}.

VII.8 Genes seleccionados que responden a la toxicidad por Mn^{+2} en nódulos de frijol común: anotación, primers y condiciones de sRT-PCR.

Abreviaturas

AGO1: Proteína Argonauta 1
ATP: Adenosin trifosfato
ASR: Arquitectura del Sistema Radicular
CAT: Catalasa
CQ: Centro quiascente
DCL1: Dicer Like protein 1
DNA: Ácido desoxirribonucleico
EST: Etiqueta de Secuencia Expresada
FSN: Fijación Simbiótica de Nitrógeno
FT: Factor de Transcripción
GC-MS: Gas Chromatografy-Mass-Spectrometry
GPX: Gauicol Peroxidasa
MAR: Meristemo Apical Radicular
NADPH: Nicotiamida- Dinucleotido fosfato.
PPi: Piro-fosfato
RNA: Ácido ribonucleico
PX: Peroxidasa
mRNA: RNA mensajero
miRNA: microRNA
Cu^{+2}: Cobre
$-Cu^{+2}$: Deficiencia de Cobre
Fe^{+2}: Hierro
$- Fe^{+2}$: Deficiencia de Hierro
Mn^{+2}: Manganeso
$+Mn^{+2}$: Toxicidad por Mn^{+2}
N_2: Nitrógeno
$-N_2$: Deficiencia de N_2
P: Fósforo
-P: Deficiencia de Fósforo
S: Azufre

RESUMEN

El frijol es una de las leguminosas más importantes en el mundo, puesto que es la principal fuente de proteínas y minerales en la dieta de los pobladores de países emergentes como México. Sin embargo, la calidad del suelo es una de las limitantes en la producción y en la calidad de la semilla de frijol. Ante esta situación, las plantas de frijol han desarrollado una variedad de adaptaciones morfológicas y bioquímicas que le permiten obtener el nutriente y/o mantener la homeostasis. En comparación a la planta modelo *Arabidopsis thaliana*, los estudios sobre la regulación a nivel transcripcional y post-trancripcional de las respuestas de frijol al estrés nutricional son limitados. En esta tesis doctoral se usaron enfoques de genómica funcional tanto bioinformáticos (cluster analysis, PathExpress y MAPMAN) como experimentales (transcriptómica por macroarreglos y quantitative-RT-PCR [qRT-PCR], Northern-Blot, silenciamiento génico por RNA de interferencia [RNAi] en plantas compuestas de frijol [plantas con raíces transgénicas y la parte aérea silvestre], metabolómica por GC-MS) para analizar las respuestas de frijol común a la deficiencia de fósforo (P) y a la toxicidad por manganeso (++Mn^{+2}). Estos análisis revelaron que ~600 y 160 genes respondieron a la deficiencia de P y toxicidad por Mn^{+2}, respectivamente. Además se identificaron las rutas metabólicas y metabolitos que se modifican ante la deficiencia de P. Se detectaron los microRNAs que responden a estrés nutricional (-P, -Fe^{+2}, -N, pH ácido y toxicidad de Mn^{+2}). Con base en los resultados obtenidos se demostró la participación del factor de transcripción PvPHR1 y el microRNA miR399 en una vía de señalización en raíces de frijol deficientes de P. Los resultados presentados en esta tesis doctoral pueden ser la base para modular la expresión de genes candidatos que respondieron a estos estreses y de esta forma mejorar el germoplasma de frijol.

ABSTRACT

Common bean (*Phaseolus vulgaris*) is one of the most important legume in Latin America and Africa; it is the principal protein and mineral source in human diet. However, the soil nutrient quality is the principal constraint for common bean production. Common bean plants have evolved several morphological and biochemical adaptation to cope with the edaphic limitation and to maintain the nutrient homeostasis. In contrast to *Arabidopsis thaliana*, model plant, the studies about the transcriptional and post-transcriptional regulation of the common bean responses to the nutrient stresses, is yet limited. In this work, different functional genomic approaches were used to analyze the transcriptional and post-transcriptional responses of common bean to phosphorus deficiency (-P) and manganese toxicity (++Mn^{+2}). Our approaches included both bioinformatic (cluster-analysis, PathExpress and MapMan) and experimental (transcriptomics through macroarray anlysis and quantitative-RT-PCR [qRT-PCR], Northern Blot, gene silencing through RNA interference [RNAi] in common bean composite bean plant [plant with transgenic roots and wild type shoot] and metabolomics through GC-MS) technologies. The transcript prifile lead us to identify ~ 600 and 160 genes that were differentially expressed in –P and ++Mn^{+2}, respectively. Also, we identified the metabolic pathways and metabolites that were modified on –P conditions. We perfomed a microRNA profile that identified 35 microRNAs that respond to nutrient stresses (-P, -Fe^{+2}, -N, acidic condition and Mn^{+2} toxicity). The results obtained led us to demonstrated the participation of the transcription factor PvPHR1 and the microRNA miR399 in a signaling pathway in P-deficient bean roots. This work provide the foundation to evaluate the individual roles of different genes (including microRNAs) and to genereate improve common bean germplasm for adaptation to nutrient stresses.

I. INTRODUCCIÓN

I.1 El frijol y el estrés nutricional

Tradicionalmente la familia Leguminosae, de acuerdo a sus características morfológicas, ha sido dividida en tres grupos o subfamilias: *Caesalpinioideae*, *Mimosoideae* y *Papilionadeae* (*Leguminosae* o *Fabaceae*). La subfamilia *Papilionadeae* es la más numerosa, puesto que está comprendida por 476 géneros y cerca de 14,000 especies. Los miembros de la familia *Leguminosae* tienen una amplia distribución que abarca desde la zona ártica hasta las zonas tropicales. Además poseen la capacidad de proliferar desde el nivel del mar hasta grandes altitudes. Su polinización es llevada a cabo por abejas, mariposas, colibríes o murciélagos, además, algunas leguminosas poseen la capacidad de auto-polinizarse. Una especie de leguminosas de gran importancia en países en vías de desarrollo es el frijol común (*Phaseolus vulgaris*), especie que pertenece a la subfamilia *Papilionoidae* de la tribu *Phaseolae* (Broughton *et al*., 2003; Doyle & Luckow, 2003; Sprent, 2008)

El frijol es uno de los cultivos más antiguos de América Latina y es extremadamente diverso en cuestiones de métodos de cultivo, usos, ambientes en los que crece y aspectos morfológicos. Al igual que otras leguminosas, el frijol puede establecer asociaciones simbióticas con bacterias fijadoras de nitrógeno (N_2) como *Rhizobium etli* y *R. tropici*. Esta interacción entre el frijol y la bacteria, después de varios eventos de comunicación entre ambos simbiontes, da como resultado la formación de órganos especializados llamados nódulos. En los nódulos se lleva a cabo la fijación simbiótica de nitrógeno (FSN) atmosférico, la cual permite que el N_2 se incorpore al metabolismo del frijol y posteriormente a la cadena alimenticia. La importancia ecológica del frijol, al igual que otras leguminosas, radica en la FSN, ya que es uno de los procesos más importantes por los cuales los mamíferos pueden obtener el N_2 e incorporarlo a su metabolismo (Broughton *et al*., 2003). Además, el frijol tiene una gran importancia económica y nutricional, puesto que esta leguminosa es una de las principales fuentes de proteínas (faseolina), vitaminas (biotina) y minerales en la dieta diaria de los pobladores de países emergentes, como México. Esta importancia alimenticia y económica es reflejada en la producción anual mundial, la cual se estima en 23 millones de toneladas métricas y en el consumo anual percápita que oscila entre 40-70 kg (Broughton *et al*., 2003)

En la actualidad, uno de los principales problemas a los que se enfrentan los productores y consumidores de frijol es la baja calidad nutricional de los suelos en los que comúnmente se cultiva el frijol. Estos suelos pueden tener una baja capacidad para retener agua, así como deficiencia de fósforo (P), hierro (Fe^{+2}) o potasio (K) y/o toxicidad por metales pesados como aluminio (Al^{+3}) manganeso (Mn^{+2}) y cobre (Cu^{+2}) (Broughton *et al*., 2003). La calidad nutricional de los suelos puede ser afectada por factores naturales y artificiales. En el primer caso se encuentra la acidez, la baja capacidad de drenado y de intercambio iónico de los suelos (Yang *et al*., 2009). En la segunda categoría destaca el mal uso de los suelos por la escasa rotación de cultivos, por el excesivo uso de fertilizantes químicos y por usar aguas negras en los sistemas de riego, las cuales pueden contener, entre otros contaminantes, altas concentraciones de metales pesados y diversos patógenos. Estos factores afectan negativamente el crecimiento del frijol, provocando baja productividad y calidad de la semilla de frijol. El crecimiento de los cultivos de frijol en condiciones de estrés ambiental se puede reproducir en condiciones de laboratorio para analizar las respuestas vegetales al estrés. En nuestro laboratorio hemos utilizado sistemas de hidroponia en condiciones

ambientales controladas (25-27 ^{0}C, 16 hrs., fotoperíodo, 60-70% humedad) para crecer plantas de frijol ante diferentes estreses nutricionales. La Figura 1 muestra el fenotipo de dichas plantas, en las que se observa que la deficiencia nutricional (-P, Fe^{+2} y –N) y el pH ácido (pH5.5) disminuyen su crecimiento. Además, las plantas deficientes de N, Fe^{+2} y con exceso de Mn^{+2} presentan hojas cloróticas. Después de varios días (3 semanas), estos estreses nutricionales inducen la senescencia y más tarde su muerte.

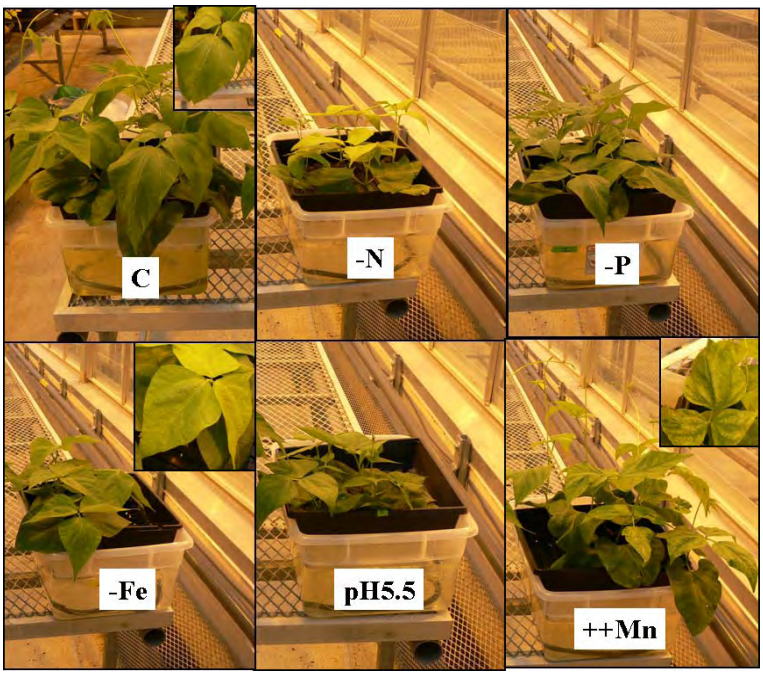


Figura 1: Efectos negativos de la deficiencia o toxicidad nutricional en el crecimiento de *Phaseolus vulgaris*. Las plantas de frijol común (*Phaseolus vulgaris*) fueron crecidas en un invernadero con condiciones ambientales controladas (25-27 ^{0}C, 16 hrs., fotoperiodo, 60-70% de humedad) durante 7 días. Se utilizó un sistema de hidroponia con la solución nutritiva Franco & Munns (Franco & Munns, 1982). Posteriormente, en los lotes experimentales, las plantas fueron sometidas durante 7 días a condiciones Control (C) o de deficiencia de N (-N), de P (-P), de Fe^{+2} (-Fe^{+2}), toxicidad de Mn^{+2} (+Mn) y a pH ácido (pH5.5) (Valdés-López *et al*., Datos no publicados)

Sin duda alguna, la deficiencia y/o toxicidad por nutrientes es una de las principales limitantes de la producción y calidad nutricional del frijol. Las deficiencias en P, Fe^{+2} y N son las que tienen mayor impacto en el desarrollo del frijol, puesto que estos tres macronutrientes son de vital importancia en el metabolismo de carbono, en la generación de biomoléculas (ATP, NADPH, fosfolípidos, DNA, RNA y proteínas), en la transferencia de energía en la cadena fotosíntetica/respiratoria de electrones, en la transducción de electrones y en la fijación de nitrógeno (Hermans *et al*., 2006; Hernández *et al*., 2009). Asimismo, la toxicidad por Mn^{+2} también es un factor limitante en el desarrollo de frijol, puesto que detiene el crecimiento de la raíz, la formación de nódulos, disminuye la fotosíntesis y la fijación de nitrógeno (González & Lynch, 1997; Valdés-López *et al*., Datos no publicados). Estos estreses nutricionales, además de afectar negativamente el crecimiento, producción y calidad de la semilla de frijol, también repercuten de la misma forma en la nutrición y economía de los países en vía de desarrollo. Asimismo, este tipo de estreses afectan negativamente la cadena alimenticia, puesto que interfiere con la obtención de la principal fuente de nitrógeno en la dieta de muchos seres vivos.

Las plantas han desarrollado diversas estrategias que le permiten responder y adaptarse al estrés nutricional. Estas respuestas van desde el nivel morfológico hasta el nivel bioquímico-molecular y pueden ser reguladas a nivel transcripcional, post-transcripcional, traduccional o post-traduccional (Rhagothama, 1999, López-Bucio *et al*., 2003; Hermans *et al*., 2006; Valdés-López & Hernández, 2008). A continuación se describirán algunas de estas respuestas al estrés nutricional, haciendo mayor énfasis en la regulación transcripcional y post-transcripcional de las respuestas a la deficiencia de P, puesto que la mayor parte de este trabajo fue realizado con el objetivo de dilucidar las adaptaciones de frijol al estrés nutricional.

I.2 El papel de la raíz en la adaptación al estrés nutricional: La participación de un programa de desarrollo post-embrionario determinado

El sistema radicular juega un papel importante en la adaptación al ambiente en el que crecen las plantas. El sistema radicular permite a la planta absorber agua y nutrientes, anclarse al sustrato y establecer asociaciones simbióticas con hongos micorrízicos o bacterias fijadoras de nitrógeno (López-Bucio *et al*., 2003). El desarrollo del sistema radicular es altamente asimétrico, lo cual refleja la plasticidad y/o capacidad de las raíces para ajustar su crecimiento y desarrollo al ambiente donde se desarrollan. Además, la arquitectura del sistema radicular (ASR) depende en gran medida del sustrato donde se encuentre (López-Bucio *et al*., 2003; Sishkova *et al*., 2007).

La disponibilidad de agua y nutrientes en el suelo es uno de los principales factores que provocan la modificación de la arquitectura del sistema radicular (López-Bucio *et al*., 2003; Hermans *et al*., 2006). Las modificaciones a la arquitectura radicular están relacionadas con el incremento de su área de exploración de regiones ricas en nutrientes y agua. Algunas de las modificaciones dependen de la especie y del ecotipo o variedad. Por lo regular, la deficiencia de nutrientes provoca un aumento en el número, longitud y densidad de raíces laterales y/o pelos radiculares, mientras que en la toxicidad nutricional el crecimiento radicular se detiene (López-Bucio *et al*., 2003; Sishkova *et al*., 2007). Existen modificaciones específicas para cada uno de estos estreses (Forde & Lorenzo, 2001; López-Bucio *et al*., 2002, 2003). Por ejemplo, en la Figura 2 se muestra que plantas de *Arabidopsis* limitadas de P (-P) detienen el crecimiento de la raíz principal y forman un gran número de raíces laterales y pelos radiculares (López-Bucio *et al*., 2002, 2003). En contraste, plantas de *Arabidopsis* deficientes de N (-N), a pesar de que la elongación de raíces laterales se incrementa, no muestra una disminución en la longitud de la raíz principal y aumento en la densidad de raíces laterales y pelos radiculares (Figura 2A) (Forde & Lorenzo, 2001; Linkohr *et al*., 2002; Tranbarger *et al*., 2003). Aunque la deficiencia de Fe^{+2} ($-Fe^{+2}$) detiene el crecimiento de la raíz principal e induce un incremento en la densidad de pelos radicales y raíces laterales, la longitud de este tipo de raíces es menor en comparación con plantas deficientes de P (Figura 2C) (Schikora & Schmidt, 2001; Schmidt & Schikora, 2001; Müller & Schmidt, 2004). Las plantas deficientes de Fe^{+2} muestran una modificación muy peculiar de este estrés, la cual consiste en la formación de invaginaciones de células epidermales y pared celular dando origen a células de transferencia (Schikora & Schmidt, 2001) (Figura 2E-F). En contraste con lo reportado para *Arabidopsis*, el análisis de raíces de plantas de frijol tratadas con –N, $-Fe^{+2}$, -P, pH 5.5 y $++Mn^{+2}$ en condiciones de hidroponia, reveló que, a excepción de las respuestas a –N, el crecimiento de la raíz principal de plantas –P y $–Fe^{+2}$ no se detiene, inclusive el número de raíces laterales es mayor en plantas $–Fe^{+2}$ que en plantas –P (Figura 3). Además, este análisis mostró que en comparación con las plantas control, el número de raíces laterales

y pelos radiculares es mayor en las tratadas con pH5.5 y ++Mn^{+2} (Figura 3) (Valdés-López, Datos no publicados).

Las diferencias en la modificación de la arquitectura del sistema radicular son debidas en parte a la molécula señal y a la batería de factores de transcripción (FT) y genes blancos que responden a cada una de estas deficiencias nutricionales. Por ejemplo, en la Figura 4 se muestra el análisis de plantas con mutaciones en genes involucrados en el control del destino celular de células de raíces (*CAPRICE, TRANSPARENT TESTA GALABRA*) y mutantes resistentes al efecto de auxinas y etileno (*axr1, axr2, ein2 y etr1*). Este análisis reveló que la producción de pelos radiculares es drásticamente afectada en plantas deficientes de Fe mientras que en plantas deficientes de P no se afectó la densidad de este tipo de raíces (Figura 4). Estos resultados demuestran que distintas vías de regulación son las encargadas en controlar las modificaciones de la raíz ante la deficiencia de P y Fe (Schmidt & Schikora, 2001; 2004; Müller & Schmidt, 2004).

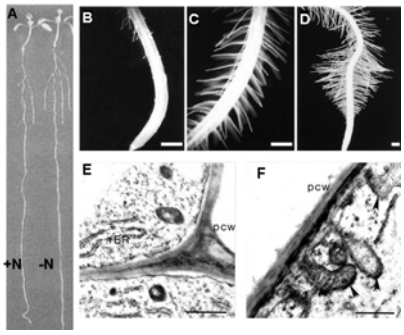


Figura 2: Producción de raíces laterales y pelos radiculares bajo condiciones de estrés nutricional. (A) Deficiencia de N. (B) Formación de pelos radiculares en condiciones nutricionales óptimas. (C) Formación de pelos radiculares en –Fe. (D) Formación de pelos radiculares en –P. (E) Células epidermales de raíces control y (F) deficientes de Fe^{+2}, las células de transferencias son indicadas por una flecha (Imágenes tomadas de: Schmidt & Schikora, 2001, 2003; Signora *et al*, 2001)

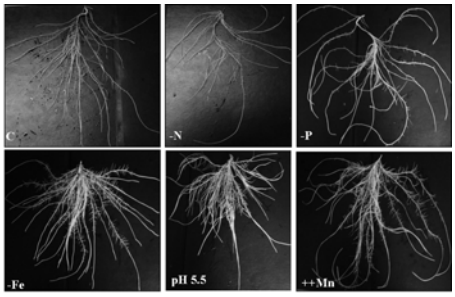


Figura 3: Las plantas de frijol modifican su arquitectura radicular de acuerdo al estrés nutricional. Raíces de plantas de frijol crecidas en condiciones de hidroponia y sometidas durante 7 días a condiciones: Control (C); deficiencia de N (-N); deficiencia de P (-P); deficiencia de Fe (-Fe^{+2}); condiciones ácidas (pH5.5), y toxicidad por Mn^{+2} (++Mn^{+2}) (Valdés-López, *et al*., Datos no publicados)

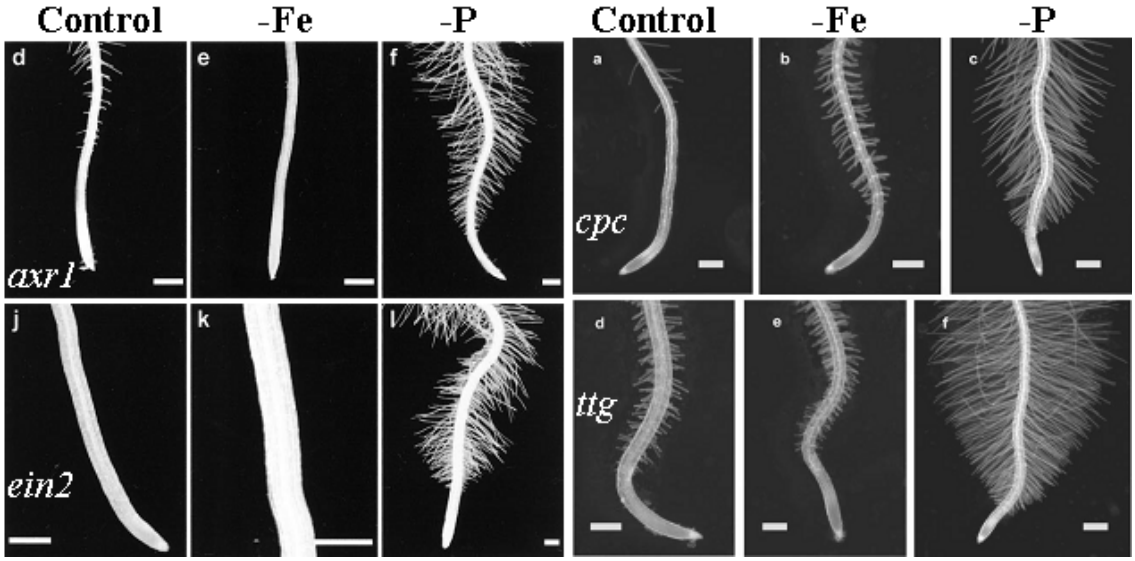


Figura 4. Evidencias de la existencia de diferentes vías de señalización en el control de la arquitectura radicular de plantas deficientes de Fe^{+2} y P. Análisis de plantas mutantes resistentes a la acción de auxinas (*axr1*), etileno (*ein2*) o con defectos en el control de la formación de pelos radiculares (*cpc: CAPRICE*; *ttg: TRANSPARENTE TESTA GALABRA*) (Imágenes tomadas de: Schmidt & Schikora, 2001; Muller & Schmidt, 2004).

Las modificaciones de la arquitectura del sistema radicular, además de ser específicas para cada deficiencia nutricional, también dependen de la especie vegetal en estudio. Por ejemplo, *Lupinus albus* (lupin) se caracteriza por tener una extrema tolerancia a la deficiencia de fósforo debido a una modificación coordinada del desarrollo y fisiología de su arquitectura radicular que conlleva a la formación de raíces proteoides (Jonson *et al*., 1996; Keerthisinghe *et al*., 1998). Las raíces proteoides, a diferencia de las raíces laterales, se desarrollan entre el eje de cada polo del xilema, además son acompañadas por el crecimiento extensivo de pelos radiculares que en conjunto resultan en un incremento de más de 100 veces de su área de absorción. Las raíces proteoides excretan ácidos orgánicos que les permiten solubilizar y absorber el fosfato de una forma más eficiente que las raíces normales, (Johnson *et al*., 1996; Keerthisinghe *et al*., 1998). Por su parte, el frijol modifica el ángulo de crecimiento y el gravitropismo de sus raíces, puesto que esta leguminosa tiende a formar raíces adventicias o raíces poco profundas, lo cual le permite explorar más fuentes de P (Bonser *et al*, 1996; Lynch & Brown, 2001; Liao *et al*., 2001). Por otro lado, las leguminosas presentan un patrón de desarrollo específico en -N. A diferencia de *Arabidopsis*, las leguminosas interaccionan con bacterias fijadoras de nitrógeno, y se ha observado que bajo estas condiciones se incrementa la formación de raíces laterales, las cuales son puntos de interacción entre la planta y la bacteria. Sin embargo, este proceso es afectado en limitación de P y toxicidad de Mn^{+2}, ya que se ha observado que en estos estreses el número de nódulos disminuye con respecto al control (Bonser *et al*., 1996; Zornosa *et al*., 2002; Carpena *et al*., 2003; Jebara *et al*., 2005; Hernández *et al*., 2009; Valdés-López, Datos no publicados).

Desafortunadamente, en frijol y en leguminosas en general, sólo se han realizado pocos estudios para identificar los genes que participan en la modificación de su arquitectura radicular. En un intento por tener evidencias genéticas de la modificación de la arquitectura radicular de frijol, se han identificado cinco QTL (Quantitative Trait Loci) en la línea endogámica recombinante (RIL) *Phaseolus vulgaris* L. (G2333/G1989), tres de ellos asociados con el número de raíces adventicias y uno con

la longitud de las raíces (Ochoa *et al*., 2006). A pesar de los esfuerzos hechos para comprender los cambios en la arquitectura radicular en frijol, la descripción a nivel molecular de las modificaciones del sistema radicular aún es limitada.

Muchos de los avances en el entendimiento de las modificaciones de la arquitectura radicular ante la deficiencia nutricional han sido realizados en la planta modelo *A. thaliana*. Por ejemplo, con este modelo se ha demostrado que bajo limitación de P el sistema radicular pasa por un programa de desarrollo post-embrionario determinado, el cual favorece la formación de nuevas raíces laterales y pelos radiculares (López- Bucio *et al*., 2002; Sánchez-Calderón *et al*., 2005; Sinhkova *et al*., 2007). Este programa inicia con la percepción de la disponibilidad del P en la rizosfera. Una vez censada la deficiencia de P, el balance hormonal del meristemo de la raíz principal es modificado a través de la acción de una oxidasa multi-cobre (MCO) que se expresa en la cofia y en el centro quiescente (CQ) de la raíz principal (Svistoonoff *et al*., 2007). Posterior a la modificación del balance hormonal, el CQ de la raíz primaria sufre alteraciones graduales que conllevan a su pérdida (Sánchez-Calderón *et al*., 2005) Asimismo, se registra una disminución en la elongación celular y una reducción progresiva de las células meristemáticas que conlleva al cese de la proliferación celular, a la pérdida irreversible del meristemo apical radicular (MAR) y, finalmente, a la disminución del crecimiento de la raíz principal (Sánchez-Calderón *et al*., 2005; Svistoonoff *et al*., 2007). Esta pérdida gradual del MAR y del CQ está mediada por los genes *LPI1* y *LPI2*, de aquí que el crecimiento de la raíz primaria no se detenga en plantas mutantes *lpi1* y *lpi2* que crecen en deficiencia de P; además, estas plantas no tienen alterados el MAR, ni el CQ (Sánchez-Calderón *et al*., 2006).

Al mismo tiempo en el que se pierde el MAR y el CQ de la raíz principal, la arquitectura del sistema radicular empieza a modificarse a través de un incremento en la formación de raíces laterales y pelos radiculares (Sánchez-Calderon *et al.,* 2005). Estos cambios son mediados por el establecimiento de gradientes de fitohormonas, así como por el incremento en la sensibilidad a auxinas (Nacry P, *et al*., 2005; Pérez-Torres *et al*., 2008). La modificación en la sensibilidad a las auxinas es mediada por el receptor de auxinas TRANSPORT INHIBITOR RESPONSE1 (TIR1), lo cual se lleva a cabo de la siguiente forma. En condiciones óptimas de fósforo (+P) los factores de transcripción ARF (Auxin Response Factors), encargados de regular la expresión de activadores/represores de genes que responden a auxinas, son regulados negativamente por la proteína AUXIN/INDOLE-3- ACETIC ACID (AUX/IAA). En limitaciones de fósforo (-P), TIR1, que forma parte del complejo de la ubiquitina ligasa SCF^{TIR1}, es inducido. Entonces, el complejo SCF^{TIR1} interacciona con la proteína AUX/IAA para llevar a cabo su degradación en el proteosoma 26S. De esta forma los factores de transcripción ARF se activan y regulan la expresión de genes que responden a auxinas, algunos de ellos encargados del desarrollo de raíces laterales, puesto que en plantas mutantes de *tir1* la formación de raíces se disminuye (López-Bucio, et. al. 2002, 2003; Pérez-Torres, 2008).

Aunque gran parte del programa de desarrollo determinado de la raíz ha sido vinculado con la acción de las auxinas, existe la posibilidad de que también participen otras hormonas como las citocininas (Dello-Ioio *et al*., 2007; Werner *et al*., 2003; Franco-Zorrilla *et al*., 2005), el etileno (López-Bucio *et al*., 2002) y el ácido giberélico (Jiang *et al*., 2007). En el caso de las citocininas, es sabido que antagonizan el papel que desempeñan las auxinas en el desarrollo de la raíz, puesto que en plantas que no acumulan citocininas, por defectos en su síntesis, se incrementa en más de siete veces el número de raíces laterales y adventicias (Werner *et al*., 2003; Miyawaki *et al*., 2006). Además, se ha reportado que para que disminuya el crecimiento de la raíz principal y la

formación de raíces laterales y adventicias es necesario que disminuya la producción de ácido giberélico (AG) y etileno (Jiang *et al*., 2007; Kim *et al*., 2008), se acumulen las proteínas DELLA y que incremente la percepción de etileno (Dello-Ioio *et al*., 2007).

Como ya se ha mencionado anteriormente, existen modificaciones en la arquitectura del sistema radicular para poder responder a un estrés nutricional dado. El incremento en la densidad de raíces laterales/pelos radiculares, así como en su longitud y distribución, permite explorar nichos ricos en nutrientes que la planta necesita para poder mantener su crecimiento y sobrevivencia. Una parte de estas modificaciones han sido explicadas en estos apartados, pero ¿cómo se modifica el flujo de carbono para mantener estas modificaciones? Una vez que la planta encuentra nichos ricos en nutrientes, ¿cómo responde la planta? ¿Cómo obtiene los nutrientes del suelo y cómo son transportados a la planta?

I.3 Respuestas metabólicas al estrés nutricional

Las plantas además de modificar su crecimiento y desarrollo, también necesitan ajustar sus procesos metabólicos, principalmente aquellos en los que participe el nutriente en deficiencia o en exceso. En cuanto a la toxicidad nutricional, la toxicidad por Mn^{+2} y Cu^{+2} se caracterizan principalmente por inhibir el transporte electrónico fotosíntetico/respiratorio y la síntesis de clorofilas (González & Lynch, 1997; Hall, 2002; Alaoui *et al*., 2004; Kepova *et al*; 2004). Este efecto negativo en el transporte electrónico conlleva a un estrés oxidativo, el cual afecta la síntesis de otras biomoléculas como lípidos, proteínas y ácidos nucleicos (Hall, 2002). Para evitar estos efectos, la planta puede expulsar el exceso de nutrientes a la rizosfera o empaquetarlos en diferentes organelos (vacuola, aparato de Golgi, retículo endoplásmico), y de esta forma mantener la homeostasis del nutriente en exceso (Pittman, 2005). Estos mecanismos de defensa y/o adaptación implican una modificación en el "flujo normal del carbono", puesto que al afectarse la fotosíntesis y la respiración mitocondrial, la producción y uso de fotosintatos disminuye (Hall, 2002; Alaoui *et al*., 2004). Ante este escenario, los fotosintatos que no se metabolizan en el ciclo de Krebs pueden servir como sustratos en la síntesis de productos de almacenaje y de ácidos orgánicos (Udhe-Stone *et al*., 2003; Vance, 2008). Las modificaciones metabólicas, al igual que en la arquitectura radicular, dependen del tipo de estrés nutricional y de la especie en estudio (Valdés-López & Hernández, 2008; Vance, 2008). Las variaciones en las modificaciones metabólicas que se dan entre diferentes especies son importantes, puesto que la capacidad que tienen para modificar su metabolismo y para mantener la homeostasis del nutriente en exceso, es lo que determina que una especie sea tolerante o sensible a la toxicidad nutricional.

Contrario a la toxicidad nutricional, en donde los excesos de los iones deben ser almacenados y/o expulsados, en la deficiencia nutricional las modificaciones metabólicas están orientadas hacia la optimización de su uso y adquisición, ya sea de reservas internas (por lo general biomoléculas) o de la rizosfera. Por ejemplo, casi todas las rutas metabólicas que requieren de P, ya sea como ión o como ATP o NADPH, son modificadas ante la deficiencia de este nutriente (Rychter *et al*., 1992; Mikulska *et. al.*, 1998). Estas modificaciones consisten principalmente en activar rutas metabólicas alternas, en las cuales se usa pirofosfato (PPi) o se evita el uso excesivo de P (Theodorou y Plaxton, 1993; Sieger, *et. al*. 2005). Algunas de las enzimas que participan en estas rutas alternas son la fosfoenol-piruvato carboxilasa (PEPc) y la malato deshidrogenasa (MDH) (Juszczuk & Rychter, 2002; Udhe-Stone *et al.*, 2003. Roux *et al*., 2005). Estas enzimas, que no requieren P, si no pirofosfato (PPi), abastecen

de fuentes de carbono al ciclo de Krebs, y además participan en la síntesis de ácidos orgánicos que son importantes en la obtención de fósforo de la rizosfera (Hernández, *et al*. 2007, 2009; Rychter *et al*., 1992; Mikulska *et al*., 1998). Además, se ha reportado que en deficiencia de fósforo la actividad de la oxidasa alternativa (AOX) es inducida (Rychter & Mikulska, 1990; Rychter *et al*., 1992. Mikulska *et al*., 1998; Sieger *et al*., 2005). Debido a que la vía alterna, mediada por la *AOX*, es una vía no fosforilativa, se cree que contribuye al consumo de oxígeno bajo condiciones prolongadas de deficiencia de Pi (Rychter & Mikulska, 1990; Rychter *et al*., 1992; Maxwell *et al*., 1999; Juszczuk, *et al*., 2001; Robson & Vanlerberghe, 2002; Vanlerberghe *et al*., 2002; Sieger, *et al*., 2005) En la Figura 5, se ejemplifican las modificaciones metabólicas que presenta *L. albus* en la deficiencia de P. En esta figura se indica en recuadros grises los genes que inducen su expresión en condiciones de -P, los cuales participan en los puntos donde se ahorra fósforo ("bypass") en la glucólisis de raíces de *L. albus* deficientes de P.

Una respuesta similar a la registrada en –P ha sido observada en la deficiencia de Cu^{+2} ($-Cu^{+2}$). En el estrés de $–Cu^{+2}$ todas las enzimas, excepto plastocianina, que requieren Cu^{+2} como cofactor, son reemplazadas por isoenzimas. Un ejemplo de este comportamiento es el documentado para la superóxido dismatasa de Cu/Zn (Cu/Zn SOD) que es reemplazada por FeSOD en condiciones limitantes de Cu^{+2} (Abdel-Ghany & Pilon, 2008; Yamasaki *et al*., 2008).

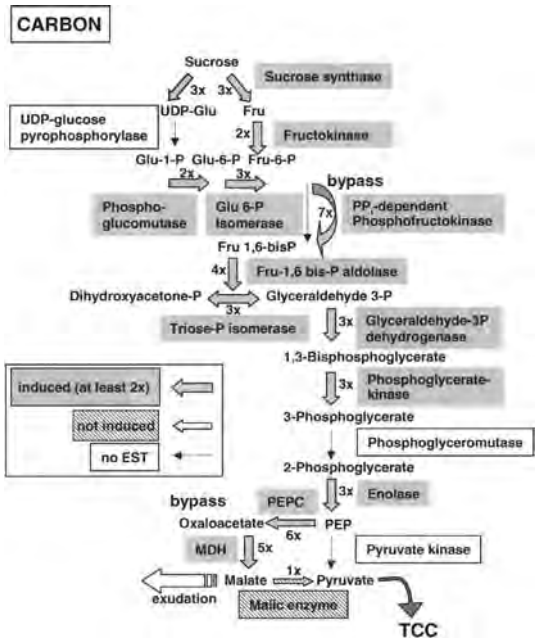


Figura 5: Modificación del flujo de carbono en raíces proteoides de *Lupinus albus* deficientes de P. Los recuadros grises indican los genes que inducen su expresión más de dos veces en este estrés. Las proteínas que codifican estos genes están involucradas en los puntos en los que se ahorra fósforo ("bypass"). (Imágen tomada de: Udhe-Stone *et al*., 2003).

Bajo condiciones de estrés nutricional, diversos azúcares son utilizados para formar productos de almacenaje, una parte de ellos son acumulados en raíces, en donde probablemente sirvan como moléculas señales o inclusive para mantener la demanda energética en la modificación de la arquitectura del sistema radicular (López-Bucio *et al*., 2002; Hermans *et al*., 2006; Amtmann & Armengaud, 2009). En raíces deficientes

de P, tanto de frijol como de *Arabidopsis*, se acumulan distintos azúcares (Almidón, sacarosa, fructosa) (Morcuende *et al*., 2007; Hernández *et al*., 2007). Sin embargo, en nódulos de frijol deficientes de P, la concentración de azúcares disminuye (Hernández *et al*., 2009). En general, en las leguminosas, las modificaciones al flujo de carbono generadas por la deficiencia de P y Fe^{+2} pueden afectar negativamente la FSN. Lo anterior se da principalmente por la reducción de las fuentes de carbono que requiere el nódulo para llevar a cabo la FSN, aunado a que este proceso requiere grandes cantidades de P y que la falta de Fe^{+2} puede afectar el complejo Fe-Molibdato de la nitrogenasa (Hernández *et al*., 2009).

Otra de las adaptaciones ante la deficiencia nutricional es la obtención de nutrientes de fuentes internas. En el caso de la deficiencia de P, una de las principales fuentes de P son los fosfolípidos y los ácidos nucleicos. Bajo estas condiciones nutricionales, tanto el mensajeroRNA (mRNA) y la actividad de fosfolipasas y ribonucleasas (RNAsas) se incrementan (Rhagotama, 1999; Valdés-López & Hernández, 2008). Debido a la degradación de los fosfolípidos de membrana, la planta los reemplaza por lípidos libres de fósforo como los glicolípidos y los sulfolípidos (Essigmann *et al*., 1998; Misson *et al*., 2005). Al respecto, diversos trabajos han reportado la inducción de genes involucrados en la síntesis de lípidos libres de fósforo (Misson *et al*., 2005; Morcuende *et al*., 2007; Hernández *et al*., 2009).

Dado que la baja disponibilidad de nutrientes en la rizosfera se debe a que varios de ellos forman complejos con otro ión, las plantas han desarrollado diversas estrategias para poder obtener los nutrientes del suelo. Entre éstas, destacan la liberación de protones, fosfatasas ácidas y el exudado de ácidos orgánicos y/o fito-sideróforos. Estos metabolitos pueden quelar (fito-sideróforos), modificar el estado iónico (Fe-quelato reductasa) y/o removilizar (fosfatasas, protones, ácidos orgánicos) los nutrientes y hacerlos disponibles a las plantas (Rhagothama, 1999; Shen, *et al*., 2002; Curie & Briat 2003; Udhe-Stone *et al*., 2003; Zhu *et al* 2005; Xiao et al., 2006; Lemanceau *et al*., 2009). La eficiencia de una planta para removilizar los nutrientes del suelo depende del tipo y la concentración de ácidos orgánicos o fitosideróforos que excretan. Por ejemplo, las raíces proteoides de *Lupinus albus* se caracterizan por excretar malato y citrato, mientras que variedades de frijol sensibles a la deficiencia de fósforo excretan tartrato y acetato, los cuales no son tan eficientes como malato y citrato para solubilizar al P (Shen *et al*., 2002; Zhu *et al*., 2005; Ryan *et al*., 2001; Roux *et al*., 2005; Uhde-Stone *et al*., 2003).

Las respuestas metabólicas para obtener el Fe^{+2} son similares a las descritas en -P (Rhagothama, 1999; Curie & Briat, 2003). Sin embargo, se han documentado dos tipos de estrategias para obtener el Fe^{+2} (Curie & Briat, 2003; Lemanceau *et al*., 2009). La estrategia I, específica para plantas dicotiledóneas, consiste en acidificar la rizosfera, mediante la excreción de ácidos orgánicos y protones. Los ácidos orgánicos interaccionan con el Fe^{+3} que se encuentra en el suelo, entonces la Fe^{+3}-quelato reductasa reduce al Fe^{+3} a Fe^{+2} y después es absorbido por la planta. En contraste, en la estrategia II, específica de monocotiledóneas, las plantas exudan diversos Fito-sideroforos, los cuales quelan al Fe^{+3} y estos complejos ($FS+Fe^{+3}$) son transportados a la planta y en el citosol los complejos $FS+Fe^{+3}$ son metabolizados para obtener el Fe^{+2} (Curie & Briat, 2003; Lemanceau *et al*., 2009).

Por último, como ya se mencionó anteriormente, el papel primordial de los ácidos orgánicos y de los fito-sideróforos es liberar el P y el Fe^{+2}, respectivamente, y hacerlo disponibles para las plantas (Rhagothama, 1999; Curie & Briat, 2003). Una vez que estan disponibles el P y el Fe^{+2}, la planta los introduce vía un sistema de cotransporte en el que están involucrados tanto transportadores de alta afinidad para Pi o

Fe, así como distintas ATPasas (Raghothama, 2000; Yan *et al*., 2002; Shin *et al*., 2004; Rubio *et al*., 2005; Shen *et al*., 2006). Distintos trabajos han demostrado que en deficiencia de P y Fe^{+2} miembros de la familia de transportadores PhT1 (phosphate high affinity transporter-1) e IRT1 (iron-regulated transporter-1), son los encargados de transportar el P y Fe, respectivamente (Rhagothama, 1999; Curie & Briat, 2003). El sistema de transporte de P en condiciones limitantes de este nutriente se da de la siguiente forma: en condiciones normales de P, PhT es regulado negativamente por la ubiquitina E3-conjugasa UBC24 (PHO2). En deficiencia de P, PHO2 es regulada negativamente a nivel post-transcripcional por el microRNA miR399, de esta forma, PhT se acumula en el citosol, donde interacciona con la proteína phosphate transporter traffic facilitator 1 (PHF1) y es transportado a la membrana plasmática. Una vez que PhT se localiza en la membrana plasmática, con la ayuda de la ATPase H^+ se lleva a cabo el co-transporte de P (Figura 6a) (Raghothama, 1999; González *et al*., 2005; Shen, *et al*., 2006). Aún en condiciones de deficiencia nutricional la planta debe mantener la homeostasis, por tanto, cuando la planta satisface sus necesidades de P, a través de un sensor aún no determinado, la planta regula el sistema de transporte de alta afinidad. Para ello, miR399 es regulado negativamente por otro riborregulador (*IPS1*), permitiendo la expresión de PHO2 y por tanto la degradación de PhT. (Bari *et al*., 2006; Franco-Zorrilla *et al*., 2007) (Figura 6b).

I.4 ¿Cómo se regulan las respuestas al estrés nutricional en plantas?

En esencia, la tolerancia de una planta a un estrés nutricional está determinada por la capacidad de la planta de mantener la homeostasis nutricional bajo estas condiciones de estrés. Dado que las adaptaciones morfológicas y bioquímicas son las que hacen que la planta pueda mantener la homeostasis, su adecuada regulación es de vital importancia. Diversos grupos de investigación han centrado sus esfuerzos en poder dilucidar las señales que desencadenan las modificaciones morfológicas/bioquímicas, así como trazar rutas de señalización y transducción que coordinan estas respuestas. Un enfoque utilizado en ello es el análisis de perfiles transcripcionales a través de microarreglos, macroarreaglos, Affimetrix Gene Chips y de secuenciación masiva (Hammon *et al*., 2003; Misson *et al*., 2005; Müller *et al*., 2007; Hernández *et al*., 2007, 2009; Calderón-Vazquez *et al*., 2008). Estos análisis han permitido identificar una gran cantidad de genes que responden a la deficiencia o toxicidad nutricional. Además, este tipo de análisis ha revelado que plantas estresadas nutricionalmente presentan dos programas transcripcionales: (1) respuesta temprana, en donde la mayoría de los genes están asociados a diversos estreses, aunque existen genes relacionados al estrés en estudio, y (2) respuestas tardías, en donde la mayoría de los genes son específicos al estrés en estudio (Misson *et al*., 2005; Valdés-López & Hernández, 2008). En los dos programas de transcripción se han identificado diversos factores transcripcionales, proteínas cinasas, genes involucrados en la modificación post-traduccional, receptores de hormonas, entre otros reguladores y probables genes blanco (Hammon *et al*., 2003; Misson *et al*., 2005; Müller *et al*., 2007; Hernández *et al*., 2007, 2009; Calderón-Vazquez *et al*., 2008). De igual forma, los diversos enfoques de secuenciación masiva, además de identificar una gran variedad de reguladores transcripcionales, traduccionales, post-traduccionales y genes blanco, han revelado la presencia de diversos RNAs que no codifican para proteínas, entre los que destacan los microRNAs (Moxon *et al*., 2008; Subramanian *et al*., 2008)

En general, con los análisis de perfiles de transcripción y de genética directa/reversa, se ha demostrado que la regulación de las respuestas al estrés

nutricional es a nivel transcripcional, post-transcripcional, traduccional y post-traduccional. Estos mecanismos de regulación pueden estar funcionando al mismo tiempo para poder coordinar finamente las respuestas al estrés nutricional. Además, diversas hormonas y azúcares juegan un papel importante en la coordinación de estas vías de regulación, puesto que pueden servir como moléculas señal. Resulta interesante que para que la planta pueda sobrevivir al estrés nutricional es necesario la participación de diversas rutas de señalización, algunas de ellas inespecíficas. Lo anterior puede deberse a que un estrés, por ejemplo deficiencia de P, puede producir diferentes moléculas señales o que la que se genera es común en varios tipos de estreses. Este fenómeno es conocido como entrecruzamiento de rutas de señalización, el cual es común tanto en estreses bióticos como en abióticos (Franco-Zorrilla *et al.,* 2005; Valdés-López & Hernández, 2008) (Figura 7). A continuación se describirá la regulación transcripcional y postranscripcional de las respuestas a la deficiencia de fósforo y se comparará con lo que se sabe hasta el momento con la deficiencia de Fe^{+2} y toxicidad por Mn^{+2}.

I.4.1. Regulación transcripcional.

Algunos de los avances sobre el entendimiento de la regulación transcripcional y traduccional de las respuestas a la deficiencia de Pi, Fe^{+2} y toxicidad por Mn^{+2} han sido realizados en *Saccharomyces cerevisae* y en algunos casos en el alga *Chlamydomonas reinhartii* (Rhagothama, 1999; Curie & Briat, 2003; Mouillon & Persson, 2006). En estos organismos se han identificado diversos componentes de las rutas de señalización (FT, transportadores, proteínas cinasas) implicadas en estos estreses (Rhagothama, 1999; Curie & Briat, 2003). Por ejemplo, en levaduras que crecen en medios libres de P y Fe, se ha observado que el control transcripcional de genes que responden a estos estreses es mediado por dos factores de transcripción, PHO82 y PHO84 en deficiencia de P, y Aft1 en deficiencia de Fe^{+2} y para algunos genes que responden a la toxicidad por Mn (Rhagothama, 1999; Curie & Briat, 2003; Lamanceau *et al*., 2009). Estos hallazgos, junto con los análisis de perfiles de transcripción, han servido de referencia en la descripción de vías de señalización en plantas.

A inicios de este siglo aún se especulaba que la regulación transcripcional de las respuestas de las plantas a la deficiencia de Pi, Fe^{+2} y toxicidad por Mn^{+2} era similar al de levaduras, sin embargo, en plantas no se han encontrado ortólogos de PHO82, PHO84 o Aft1. Lo anterior sugería que las plantas habían desarrollado un sistema diferente y quizá más complejo en el que podrían estar participando más de dos TF. Esta hipótesis fue reforzada al analizar los perfiles transcripcionales de plantas estresadas nutricionalmente, lo cual reveló que varios factores de transcripción respondían a estos estreses (Misson *et al*., 2005; Hernández *et al*., 2007, 2009; Calderón-Vázquez *et al*., 2008). Estas evidencias sugerían que el control transcripcional de las respuestas a la deficiencia nutricional era mediado por varios factores de transcripción y que probablemente distintas vías podían participar en la regulación de las respuestas a un estrés determinado.

Los avances en el entendimiento de la regulación transcripcional de las respuestas de las plantas a la deficiencia de fósforo se iniciaron al descubrir y caracterizar la proteína PHR1 (phosphate high response-1), el primer FT vinculado a este estrés (Rubio *et al*., 2001). PHR1 es un ortólogo de IPSR1 de *C. reinhartii* y se caracteriza por tener dos dominios, el dominio MYB y el coiled-coil. Este FT es modificado post-traduccionalmente por sumolización, llevada a cabo por la proteína SUMO E3 ligasa que es codificada por el gen *SIZ1*, lo cual determina su localización nuclear (Miura *et al*., 2005). PHR1 se une como dímero al palíndrome imperfecto GNATATNC, el cual se encuentra en la región promotora de los genes que responden específicamente a la deficiencia de P (Rubio *et al*., 2001). La respuesta de PHR1 a la deficiencia de P es baja,puesto que la inducción en sus niveles de expresión es de tan solo 2 veces (Rubio *et al*., 2001). Sin embargo, se tiene reportado que este FT regula positivamente la transcripción de genes involucrados en el flujo de carbono (*PPCK*), en la síntesis de antocianinas (*CHS*), así como en la removilización (*RNAse, APs, APC5*), transporte (*Pht1, PHO1,H10*) y en la homeostasis (*At4, IPS1*, miR399) de P (Bari *et al*., 2006; Chen *et al*., 2007; Nilson *et al*., 2007; Rubio *et al*., 2001; Stefanovic *et al*., 2007). El análisis de plantas mutantes de *A. thaliana phr1,* reveló que eran hipersensibles a la deficiencia de P, puesto que varios de sus genes blancos no respondían a los bajos niveles de P; a pesar de ello, la arquitectura radicular se modificó igual que la de las plantas silvestres (Rubio *et al*., 2001). Lo anterior demuestra que PHR1 no regula la modificación de la arquitectura radicular. En general, esta vía opera de la siguiente forma: después de percibir la deficiencia de P, PHR1 es translocado al nucleo, vía SIZ1, y reconoce el palíndrome inperfecto GNATATNC que se encuentra en la región promotora de genes que están asociados a la removilización, transporte, translocación y homeostasis de P.

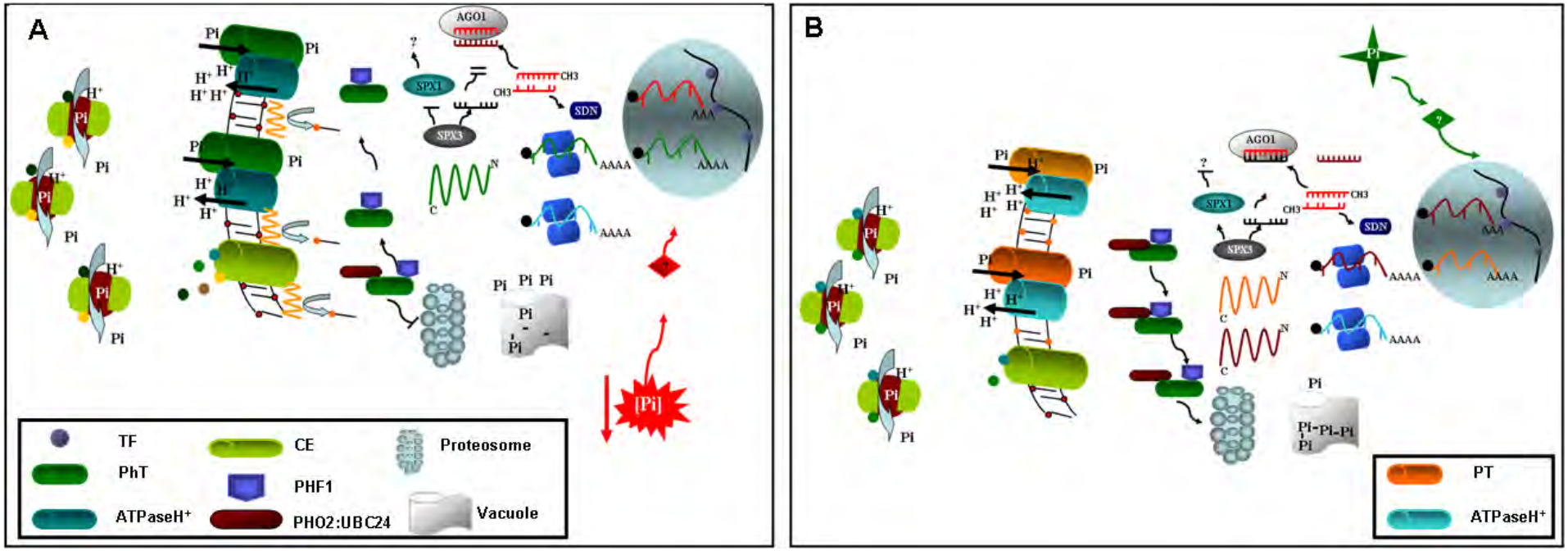


Figura 6: Sistema de regulación de la homeostasis de P en plantas dicotiledóneas. (A) En condiciones limitantes de P el sistema de transporte de alta afinidad a P es activado, el cual es regulado a nivel transcripcional, post-transcripcional y post-traduccional (ver detalles en el texto). Asimismo, en estas condiciones la participación de distintas fosfatasas (representadas en líneas de color naranja) y la secreción de ácidos orgánicos (representados por círculos en distintos colores) es importante para obtener el P de los fosfolípidos de membrana y para solubilizar el P que se encuentra en la rizosfera, respectivamente. (B). Una vez que la planta satisface sus necesidades de P, el sistema de transporte de alta afinidad de P es regulado negativamente a través de la acción de la ubiquitina UBC24, de la participación del riboregulador IPS1 y de SPX3 (ver detalles en el texto) (Esta figura fue construida de acuerdo a la información publicada por: González *et al*., 2005; Bari *et al*., 2006; Franco-Zorrilla *et al*., 2007; Duan *et al*., 2008; Voinnet *et al*., 2009; Wang *et al*., 2009).

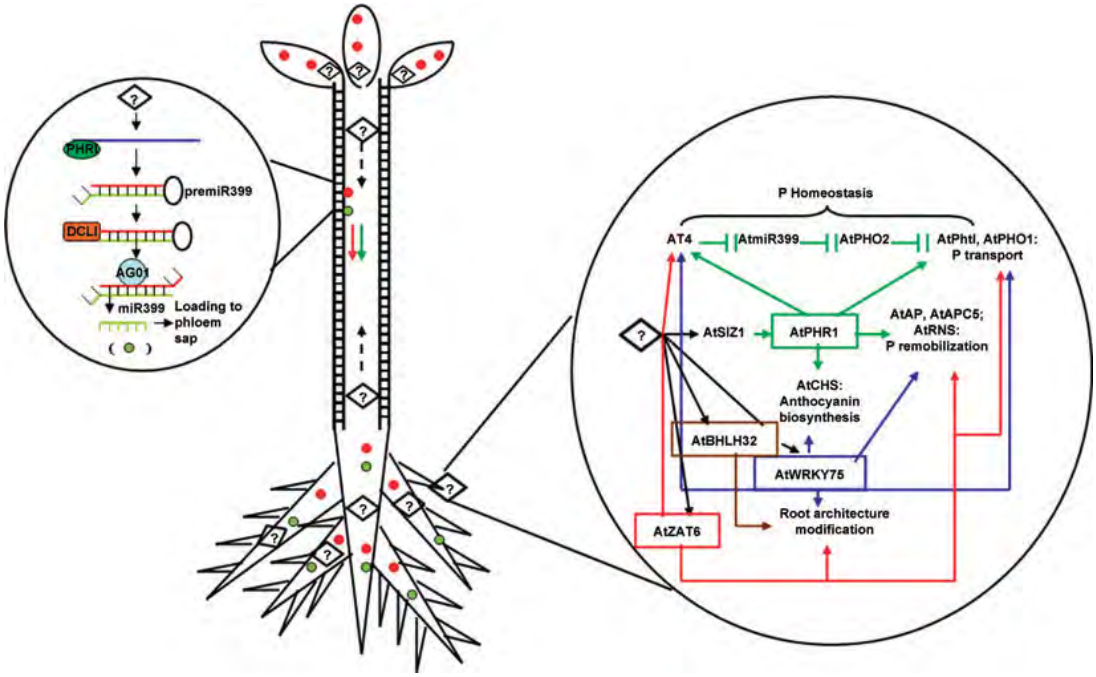


Figura 7: Modelo para la vía de señalización de deficiencia de P en *A. thaliana*. En este modelo se representa el entrecruzamiento de rutas de señalización que se da en las respuestas a la deficiencia de P. Los círculos rojos y verdes, representan azúcares y miR399, respectivamente. El rombo con un signo de interrogación, indica una molécula señal desconocida. (Imagen tomada de Valdés-López & Hernández, 2008)

Primero, miR399 reconoce al mRNA de PHO2 y dirige su corte en el Complejo de Silenciamiento de RNA (RISC), lo cual permite que los transportadores de alta afinidad (Pht1) se localicen en la membrana y transporten el fósforo que fue removido de la materia orgánica de la rizosfera por distintos ácidos orgánicos. En el citoplasma diversas fosfatasas ácidas, controladas por PHR1, obtienen el P de distintas biomoléculas. El fósforo es translocado a distintas partes de la planta a través de PHO1;H10. Una vez que la planta satisfizo sus necesidades de P, *IPS1* secuestra a miR399 y lo inactiva, permitiendo de esta forma a PHO2 dirigir al Pht1 al proteosoma y reestablecer el sistema de baja afinidad a P. Este proceso es ilustrado en la Figura 6.

Como ya se ha mencionado anteriormente, la sobrevivencia de una planta ante un estrés nutricional está determinada en parte por su capacidad de mantener la homeostasis. La vía de señalización de PHR1 es la encargada de regular la homeostasis (Rubio *et al*., 2001; Bari *et al*., 2006). Aún en el año 2007 se aceptaba que este proceso era regulado sólo por UBC24, PhT1, miR399 e IPS1. Sin embargo, recientemente se han aportado evidencias de la participación de miembros de la sub-familia génica SPX en el control de la homeostasis de P (Duan *et al*., 2008; Wang *et al*., 2009). Los 3 miembros de esta sub-familia (*SPX1*, *SPX2* y *SPX3*) se inducen en condiciones limitantes de P y son regulados positivamente por PHR1 (Duan *et al*., 2008). Aunque estos tres genes bajo condiciones deficientes de P se expresan principalmente en raíces, ambos presentan diferente localización subcelular, dos ellos (*AtSPX1* y *AtSPX2*) se localizan en el núcleo, mientras que el tercero (*AtSPX3*) se localiza en el citoplasma (Duan *et al*., 2008). Resulta interesante que a pesar de que estos genes no presentan

dominios de unión al DNA, ellos regulan la expresión de genes involucrados en la removilización, transporte y homeostasis de P (Duan *et al*., 2008; Wang *et al*., 2009). El papel de SPX en la regulación de la homeostasis no es claro, sin embargo, se propone que participen en una regulación tipo "feedback" como se ha reportado en levaduras, en las cuales SPX regula la velocidad del transporte de P (Duan *et al*., 2008; Hüliman *et al*., 2009; Wang *et al*., 2009). Los resultados presentados por Duan y colaboradores (2008) sugieren que AtSPX3 es un regulador negativo de AtSPX1. Asimismo, AtSPX1 podría ser un regulador positivo de los genes *IPS1, AT4, PhT1;4; PhT1;5, ACP5* y *RNS*, puesto que plantas SPX3-RNAi muestran un incremento en los niveles de SPX1 y de sus probables genes blanco (Duan *et al*., 2008) (Figura 6). Desafortunadamente, no existen datos que demuestren la interacción de SPX1 con los genes blanco, o con otros miembros de esta sub-familia. Por otro lado, en levadura se sabe que miembros de la sub-familia SPX funcionan como sensores de la señal de deficiencia de fósforo (Lenburg & O'Shea, 1996; Mouillon & Persson, 2006). En plantas, es probable que SPX3 pueda ser el censor de una señal que desencadene las respuestas a la deficiencia de P a nivel sistémico. Lo anterior debido a que la inducción de algunos genes blanco en tallos no es la misma que la registrada en raíces, lo cual permite especular que la percepción de la señal de deficiencia de P es censada de distinta forma en cada uno de estos órganos.

La vía de señalización mediada por PHR1 ha sido identificada en arroz y en frijol común, lo cual sugiere que está conservada en monocotiledóneas y dicotiledóneas (Valdés-López *et al*., 2008; Zhou *et al*., 2008). Sin embargo, aunque en monocotiledóneas miR399 se acumula en deficiencia de P, en estas plantas la homeostasis no es mediada por miR399 como en *Arabidopsis* y frijol (Valdés-López *et al*., 2008; Zhou *et al*., 2008). En arroz, la homeostasis es regulada por OsSPX1 en un proceso similar al descrito anteriormente (Figura 6) (Wang *et al*., 2008).

Además de la vía de PHR1, en *Arabidopsis* se han descrito otras cuatro rutas de señalización, las cuales están reguladas por miembros de la familia WRKY (WRKY75), Zinc Finger Protein (ZAT6), MYB (MYB62) y bHLH (bHLH32) (Chen *et al.*, 2007; Devaiah *et al*., 2007a, 2007b, 2009). Los miembros de estas familias que responden a la deficiencia de fósforo están involucrados en la modificación de la arquitectura radicular y en la re-movilización y transporte de P. Resulta interesante que algunos de los genes blanco, principalmente los de re-movilización y transporte, también son regulados por PHR1 (Rubio *et al*. 2001). Lo anterior demuestra que la regulación de las respuestas a la deficiencia de P es mediada por varios FT y rutas de señalización.

Hasta el momento se ha reportado la participación de dos miembros de la familia bHLH en la regulación de las respuestas a la deficiencia de fósforo (Yi *et al*., 2005; Chen *et al*., 2007). Estos miembros son *OsPTF1* y *AtbHLH32*, los cuales son inducidos y reprimidos en plantas de arroz y *Arabidopsis* deficientes de P, respectivamente (Yi *et al*., 2005; Chen *et al*., 2007). Análisis de mutantes de OsPTF1 y AtbHLH32 revelaron que estos FT están involucrados en el control de la longitud total de la raíz y en la formación de pelos radiculares, respectivamente (Yi *et al*., 2005; Chen *et al*., 2007). Al respecto, se ha reportado que AtbHLH32 interactúa con TTG1 (TRANSPARENT TESTA GLABRA1), una de las proteínas involucradas en definir el destino celular de las células de la raíz, (Chen *et al*., 2007). bHLH32, además de regular genes involucrados en la plasticidad de la raíz, también regula negativamente a PPCK en condiciones óptimas de P. PPCK, bajo condiciones deficientes de P, es regulada positivamente por AtPHR1, y es la encargada de fosforilar a PEPC, la cual, a su vez, se encarga de llevar a cabo una de las vías glucolíticas alternas y en la síntesis de ácidos orgánicos, dos procesos que son inducidos en limitación de Pi (Chen *et al*., 2007;

Vance, 2008). Por otro lado, OsPTF1 reconoce cajas G (CAGTG) y E (CANNTG), las cuales se han identificado *in silico* en las regiones promotoras de los genes que codifican para ATPasa H^+; pirofosfatasas H^+ vacuolares; proteínas asociadas a senescencia; cinasas; varios citocromos P450, transportadores ABC y RNasa, estos dos últimos también regulados positivamente por PHR1 en *A. thaliana* (Yi *et al*., 2005; Valdés-López *et al*., 2008).

Algunos miembros de la familia de FT bHLH también están involucrados en la regulación de las respuestas a la deficiencia de Fe (Curie & Briat, 2003; Lemanceau *et al*., 2009). Los miembros de esta familia que responden a la deficiencia de Fe son: FIT1 (Fe-deficiency induced transcription factor 1), bHLH029, bHLH38 y bHLH39 (Jakoby *et al*., 2004; Colangelo & Guerinot, 2004; Ogo *et al.,* 2008). Se ha demostrado que estos FT interactúan entre sí para formar un complejo proteíco, el cual regula positivamente la expresión de transportadores de alta afinidad a Fe (IRT), Fe-quelato reductasa (FRO) y de otros genes involucrados en la homeostasis de Fe (Curie & Briat, 2003; Lemanceau *et al*., 2009)

Además de la participación de los FT bHLH en el desarrollo de la raíz bajo deficiencia de Pi, se ha reportado que *AtWRKY75* y *AtZAT6* también regulan los cambios en la arquitectura radicular, puesto que plantas de *A. thaliana* WRKY75-RNAi o ZAT6 sobre-expresado (ZOe) muestran un incremento en la formación de raíces laterales y en la disminución del crecimiento de la raíz, respectivamente (Devaiah, *et al*., 2007a, 2007b). Los cambios observados en la arquitectura radicular de estas mutantes fueron registrados tanto en condiciones óptimas y deficientes de Pi, por lo que Devaiah, *et al*. (2007a, 2007b), propone que las modificaciones al sistema radicular son independientes a los niveles de Pi que tenga la planta completa. Sin embargo, este posible comportamiento se contrapone a lo observado en otros trabajos, en los cuales se ha demostrado que el programa de desarrollo determinado de la raíz bajo deficiencia de Pi depende de los niveles de Pi tanto intracelular como los que haya en el suelo (Sánchez-Calderón, *et al*., 2005, 2006; Svistoonoff, *et al*., 2007; Vance, 2008).

WRKY75 además de participar en la modificación de la arquitectura radicular, también regula positivamente los genes que codifican para las fosfatasas ácidas: *AtPS2-1*, *AtPS2-2*, *AtPS2-3*, para dos RNAs no codificantes: *AT4*, *AtIPS1*, y para transportadores de alta afinidad por Pi: *Pht1;1* y *Pht1;4*; los cuales también son regulados positivamente por PHR1, (Devaiah *et al*., 2007a; Rubio *et al*., 2001). Las regiones promotoras de estos genes poseen cajas W, reconocidas por WRKY75, y el palíndrome imperfecto GNATATNC, reconocido por PHR1 (Devaiah *et al*., 2007a; Rubio *et al*., 2001). Debido a que algunos de los genes que regula WRKY75 están involucrados en la re-movilización y en el transporte de Pi, este TF, al igual que PHR1, regula el transporte y homeostasis de Pi (Devaiah *et. al*., 2007a; Rubio *et. al.,* 2001). Estos datos permiten especular que estas vías (WRKY75 y PHR1) se complementan una a la otra cuando alguna de ellas está afectada, o inclusive ambas vías pueden tener un efecto sinérgico.

La participación del FT MYB62 en la regulación de las respuestas a la deficiencia de P es interesante, puesto que además de participar en la modificación de la arquitectura radicular, también participa en el control del crecimiento vegetativo, así como en la floración (Devaiah *et al*., 2009). El papel de MYB62 consiste en regular negativamente genes que intervienen en la biosíntesis de ácido giberélico (*CPS, KS, KO, GA20ox1*) y floración (*SUPPRESOR OF CONSTANS 1: SOC1*; *SUPERMAN: SUP*). La represión de genes asociados a la síntesis de ácido giberélico favorece la disminución del crecimiento de la parte área de la planta, lo cual es una respuesta característica en la deficiencia de P (Rhagothama, 1999; Vance, 2008).

Por otro lado, es importante mencionar que los análisis de plantas de *Arabidopsis* que sobre-expresaban por separado los FT ZAT6 y MYB62 mostró que la expresión de varios genes blanco involucrados en la re-movilización, transporte y homeostasis de P, se disminuyó (Devaiah *et al*., 2007b, 2009). Estas observaciones sugieren que el nivel de expresión de cada uno de los FT que responden a la deficiencia de P debe ser finamente regulada y que cualquier modificación puede afectar negativamente la homeostasis del P.

La regulación transcripcional de las respuestas a la toxicidad por Mn^{+2} aún no ha sido estudiada ni en *Arabidopsis* ni en otras plantas. La mayoría de los avances en este tema han sido orientados en identificar sistemas de transporte que permitan regular la homeostasis de Mn^{+2}, así como las respuestas antioxidativas (Pittman, 2005).

En general, en leguminosas la información sobre la regulación transcripcional de las respuestas al estrés nutricional es limitada; hasta el momento se conocen algunos de los factores de transcripción que responden a la limitación de fósforo y toxicidad por Mn (Hérnandez *et al*., 2007, 2009; Valdés-López *et al*, datos no publicados), sin embargo, solo se ha analizado el papel fisiológico del FT MYB (PvPHR1) que responde a la deficiencia de P (Valdés-López *et al*., 2008).

I.4.2. Participación de los miRNAs en la regulación de las respuestas a la deficiencia nutricional.

Los miRNAs son RNAs que no codifican para proteínas y su longitud es de 18-24 nts (Jones-Rhoades *et al*., 2006; Voinet, 2009). La biogénesis de los miRNAs es llevada a cabo en el núcleo y en el citoplasma de la siguiente forma: los microRNAs primarios (pri-miRNAs), de longitud de aproximadamente 1000 pb, interaccionan con la proteína DAWDLE (DDL) (Yu *et al*., 2008). El complejo Pri-miRNA-DDL interacciona con otro complejo proteíco conformado por las proteínas Dicer-Like1 (DCL1), Serrate (SE) y HYL1. Este complejo proteíco procesa al pri-miRNA para dar lugar al pre-miRNA, el cual tiene una longitud variable y menor que la del pri-miRNA, además se caracteriza por tener una estructura de tallo y asa (Jones-Rhoades *et al.*, 2006; Voinnet, 2009). El pre-miRNA es cortado por DCL1 y da lugar a un RNA de doble cadena (18-24 nt), el cual es metilado por HEN1 y exportado al citoplasma donde la proteína Argonauta 1 (AGO1) selecciona al miRNA maduro, mientras que el miRNA* es degradado por la RNAse SDN (Ramachandra & Chen, 2008). El miRNA maduro es cargado a AGO1, lo cual da origen al complejo de silenciamiento inducido por RNA (RISC) (Voinnet, 2009). Los miRNAs interaccionan con mRNAs y regulan su expresión, ya sea a nivel post-transcripcional (corte del mRNA) o a nivel traduccional inhibiendo la traducción del mRNA (Voinnet *et al*., 2009). Hasta hace poco tiempo se aceptaba que la mayoría de los miRNAs de plantas regulaban sus blancos a través del corte de sus mRNAs blancos, sin embargo, en 2008 y 2009 se aportaron evidencias, tanto bioquímicas como genéticas, de que los miRNAs de plantas también regulan sus mRNAs blancos a nivel traduccional (Brodersen *et al*., 2008; Lanet *et al.*, 2009).

Existe una gran cantidad de evidencias que vinculan a los miRNAs con el control del crecimiento y desarrollo de la planta en condiciones normales (John-Rhoades *et al*., 2006; Mallory & Vaucheret, 2006). Se ha reportado que miR166 y miR169 participan en el desarrollo de nódulos en *Medicago truncatula* (Combier *et al*., 2006; Boualem *et al*., 2008). Asimismo, se ha documentado la participación de los miRNAs en la adaptación a diferentes estreses bióticos y abióticos. Por ejemplo, miR169/miR396/pvu-miR2118/pvu-miR159.2, miR393, miR395, miR167, miR397/miR398/miR408/miR857, y miR399/miR144/miR827 están involucrados en

sequía, patogénesis, en deficiencia de N, azufre (S), Cu^{+2} y de P, respectivamente (Li *et al*., 2008; Liu *et al*., 2009; Arenas-Huertero *et al*., 2009; Navarro *et al.*, 2006, Bari *et al*., 2006; Pant *et al*., 2009). Algunos miRNAs involucrados en el estrés nutricional regulan la expresión de genes asociados a la regulación de la homeostasis nutricional (Bari *et al*., 2006; Yamasaki *et al*., 2008; Kawashima *et al*., 2009). Por ejemplo, en deficiencia de Cu^{+2}, las proteínas de los mRNA blancos de miR398 (*Cu/Zn-SOD*) y miR397/miR408/miR857 (*Lacasa*) requieren una gran cantidad de Cu^{+2} para llevar a cabo sus reacciones y se propone que su expresión es regulada para "ahorrar" el poco Cu^{+2} que tiene la planta (Yamasaki *et al*., 2008; Abdel-Ghany & Pilon, 2008). Cabe mencionar que existen miRNAs que, aunque responden a la limitación nutricional, no necesariamente están involucrados en la homeostasis. Por ejemplo, a diferencia de miR399, el cual sí regula la homeostasis de P en dicotiledóneas (revisado en el apartado I.3 de este trabajo), en deficiencia de P miR857 está involucrado en la acumulación de antocianina (Pant *et al*., 2009), una respuesta muy característica de este estrés. A pesar del gran número de secuencias de miRNAs obtenidas por secuenciación masiva, no se han identificado miRNAs que estén asociados con las modificaciones morfológicas que experimenta la planta durante un estrés nutricional.

Uno de los grandes retos en los estudios de las respuestas a los estreses nutricionales es la identificación de las moléculas señal que desencadenan los cambios moleculares, bioquímicos y morfológicos. Recientemente se planteó la idea de que los miRNAs pueden ser moléculas señalizadoras móviles en la deficiencia nutricional, puesto que se propone que miR395 y miR399, involucrados en la regulación de la homeostasis, son sintetizados en órganos distintos de aquéllos donde realizan su función (Pant *et al*., 2008). Aunque no se ha dilucidado el sistema de transporte sistémico de los miRNAs, grupos de investigación que apoyan está hipótesis sugieren que los miRNAs pueden regular las asociaciones simbióticas que le permite a la planta obtener ciertos nutrientes (Pant *et al*., 2008, 2009). Por ejemplo, se plantea que miR169 puede participar en la regulación sistémica de la nodulación en leguminosas, puesto que tiene como blanco a *CLAVATA-Like1*, FT involucrado en este proceso (Pant *et al*., 2009). Sin embargo, aún faltan evidencias que demuestren que realmente los miRNAs son moléculas señalizadoras móviles.

Al igual que otros genes que responden al estrés nutricional, los miRNAs deben ser finamente regulados, puesto que un cambio en la regulación de su expresión puede afectar drásticamente el desarrollo y la sobrevivencia de la planta. Los miRNAs pueden ser regulados transcripcionalmente por FT, puesto que en la región promotora de los miRNAs se ha encontrado distintos elementos *cis* característicos de algunos estreses nutricionales (Yamasaki *et al*., 2009). Algunos miRNAs pueden ser regulados a través de un proceso de mimetismo, el cual involucra a un pseudo-blanco que secuestra al miRNA, y de esta forma regula su acción sobre el verdadero blanco (Franco-Zorrilla *et al*., 2007). Un tercer mecanismo podría ser la degradación de los miRNAs maduros por medio de una RNAsa (SDN) específica para este tipo de RNAs, sin embargo, no existen estudios que vinculen la expresión de SDN en condiciones de estrés (Ramachandran & Chen., 2008).

Enfoques recientes de secuenciación masiva han identificado varios miRNAs específicos para leguminosas, como *Medicago truncatula*, *P. vulgaris* y *Glycine max* (Szittya *et al*., 2008; Arenas-Huertero *et al*., 2009; Subramanian *et al*., 2008). Sin embargo, no existen reportes de la participación de miRNAs en la regulación de las respuestas de frijol a la deficiencia nutricional. Aunado a lo anterior, aún en plantas modelo, no existen reportes de la probable participación de miRNAs en la adaptación de plantas deficientes de Fe^{+2} o estresadas por toxicidad por Mn^{+2}.

II. OBJETIVOS

A pesar de la importancia económica, ecológica y alimenticia que tiene el frijol, existen pocos estudios sobre la regulación transcripcional y post-transcripcional de las respuestas de esta leguminosa a la deficiencia de P y a la toxicidad por Mn^{+2}. Por lo anterior se plantearon los siguientes objetivos:

Objetivo general:

Analizar a nivel transcripcional y post-transcripcional las respuestas de frijol común al estrés nutricional.

Objetivos particulares:

- Analizar las respuestas transcripcionales de frijol común a la deficiencia de fósforo y toxicidad por manganeso
- Identificar los factores de transcripción y genes blanco que respondan a la deficiencia de P y toxicidad de Mn^{+2}.
- Identificar los miRNAs que respondan a la deficiencia de P y otros estreses nutricionales.
- Trazar por lo menos una vía de señalización de las respuestas de frijol común a la deficiencia de P o toxicidad de Mn^{+2}.

II.1 Estructura de la Tesis

En esta tesis se presentan resultados que contribuyen al conocimiento de la regulación a nivel transcripcional y post-transcripcional de las respuestas de frijol a la deficiencia de fósforo y toxicidad por Mn. En el primer trabajo (Anexo VII.1), se identificaron bioinformáticamente 52 genes que responden a la deficiencia de fósforo en frijol común. Algunos de los genes identificados fueron ribonucleasas, fosfatasas, diferentes transportadores y factores de transcripción. Este trabajo sirvió de referencia para poder identificar experimentalmente los FT y genes blanco involucrados en la regulación de las respuestas de frijol a este estrés.

En el segundo y tercer trabajo (Anexo VII.2 y VII.3) se analizó el perfil transcripcional y el metaboloma de raíces y nódulos de plantas de frijol deficientes de P. En estos dos trabajos se identificaron alrededor de 500 genes que responden a la deficiencia de P. En estos dos trabajos se identificaron diversos FT, genes asociados a las respuestas morfológicas y bioquímicas, así como las rutas metabólicas y los metabolitos que se modifican en este estrés. Es importante mencionar que varios de los genes identificados con estos enfoques experimentales también fueron detectados en nuestros análisis bionformáticos.

El cuarto trabajo (Anexo VII. 4) es una revisión sobre la regulación transcripcional y post-transcripcional de las respuestas de leguminosas a la deficiencia de fósforo. En él se revisan los avances sobre leguminosas y se discuten las diferencias en las repuestas de estas plantas con respecto a *Arabidopsis* y arroz.

En el quinto trabajo (Anexo VII. 5) se describe una ruta de señalización de las respuestas a la deficiencia de fósforo en raíces de frijol. En él se describe el papel del FT PvPHR1 y del microRNA pvu-miR399 en la adaptación de frijol a este estrés nutricional.

En el sexto trabajo (Anexo VII. 6) se identificaron algunos de los miRNAs que responden a la deficiencia de P, Fe, N, pH ácido y toxicidad de Mn^{+2} en frijol. Se

analizó la expresión de 70 miRNAs en hojas, raíces y nódulos de frijol de plantas controles y deficientes de P, Fe^{+2} o N, así como en exceso de Mn^{+2} y en pH ácido.

Finalmente, en el Apartado VII.7 de esta tesis se describen los resultados obtenidos en el análisis transcripcional y bioquímico de las respuestas de nódulos de frijol a la toxicidad por Mn^{+2}. En este trabajo se identifican algunos genes blanco, FT, miRNAs y enzimas anti-oxidativas que responden en nódulos a la toxicidad por Mn.

III. Resultados

III.1 Deficiencia de fósforo

III.1.1 Identificación *in silico* de genes que responden a la deficiencia de fósforo en frijol

Los primeros resultados de esta tesis doctoral se encuentran detallados en el Anexo VII.1, "Identification of candidate phosphorus stress induced genes in *Phaseolus vulgaris* through clustering analysis across several plant species" por Michelle A. Graham, Mario Ramírez, Oswaldo Valdés-López, Miguel Lara, Mesfin Tesfaye, Carroll P. Vance y Georgina Hernández, publicado en *Functional Plant Biology* 33: 789-797 (2006).

En este artículo se reporta la elaboración de un "Gene Index" de *Phaseolus vulgaris*, el cual consistió en el ensamblaje de 20, 578 EST en "Contigs" y "Singletons". Las secuencias de ESTs fueron publicadas por Ramírez *et al*., 2005 y Melotto *et al*., 2005. De estas secuencias, 6, 787 EST fueron clasificados como "Singleton", mientras que 13, 791 EST fueron ensambladas en 2, 883 "Contigs", dando como resultado 9, 670 genes únicos.

Con el propósito de identificar genes relevantes en la adaptación de frijol a la deficiencia de P, en este trabajo se realizó un análisis de tipo "Clustering", con base en bibliotecas de cDNA de órganos deficientes de fósforo de las leguminosas: *Phaseolus vulgaris*, *Lupinus albus*, *Medicago truncatula*, *Glycine max*, y *Arabidopsis thaliana*. En un primer análisis se identificaron "Contigs" sobre-representados estadísticamente en las bibliotecas de órganos deficientes de P con respecto a órganos control. El número de contigs sobre-representado en condiciones deficientes de P fueron: 247 en *P. vulgaris*, 543 en soya, 404 en *M. truncatula*, 494 en *A. thaliana* y 409 en *L. albus*. Los contigs estadísticamente sobre-representados en cada especie fueron utilizados en un análisis bioinformático tipo "Single-linkage clustering", con el cual se identificaron 22 grupos de genes que estaban sobre-representados en las 5 especies analizadas. Secuencias de *P. vulgaris* fueron ubicadas en 19 de estos 22 grupos. Combinando estos análisis bioinformáticos con datos disponibles de micro y macroarreglos, se identificaron 52 secuencias de *P. vulgaris* que puedieran participar en la adaptación de esta leguminosa en la deficiencia de P. Algunos de los genes identificados fueron: fosfatasas, RNAsas, distintas cinasas, factores de transcripción y peroxidasas. Los resultados de este trabajo fueron la base para futuros análisis, como el análisis transcripcional de raíces y nódulos de plantas de frijol deficientes de P.

III.1.2 Análisis transcripcional de las respuestas de raíces de frijol a la deficiencia de fósforo

Los resultados del análisis transcripcional y metabólico de las respuestas de raíces de plantas frijol común deficientes de fósforo se encuentran detallados en el

Anexo VII.2: “Phosphorus stress in common bean: root transcript and metabolic responses”, por: Georgina Hernández, Mario Ramírez, Oswaldo Valdés-López, Mesfin Tesfaye, *et al*., publicado en *Plant Physiology* 144: 752-767 (2007).

En este trabajo las plantas de frijol (cv. Mesoamericano Negro Jamapa) se crecieron por 21 días en macetas con vermiculita regándose con una solución nutritiva con 200 veces menor concentración (5μM) de P que la solución control (1mM). Se analizaron algunos parámetros fisiológicos con el propósito de verificar que nuestras condiciones experimentales utilizadas inducían respuestas características a la deficiencia de P. Los contenidos de P de las plantas estresadas mostraron una disminución de más del 50% con respecto al control. Además, las plantas deficientes de P incrementaron la proporción raíz/tallo (peso seco). Asimismo, se registró una reducción en la fotosíntesis neta, principalmente en los eventos de carboxilación y en la regeneración de la ribulosa 1,5 bisfosfato. Estos parámetros fisiológicos demostraron que las plantas de frijol se encontraban estresadas por deficiencia de P, por lo que se procedió a analizar el perfil transcripcional de raíces de estas plantas. El análisis transcripcional, vía hibridación de macro-arreglos impresos con 2, 212 genes únicos de raíces de frijol deficientes de P, reveló que 126 genes respondieron a la deficiencia de P en raíces de frijol. De éstos, 78 genes fueron inducidos de dos a siete veces, y el resto de los genes (48) mostraron una represión de su expresión de 2 a 5.88 veces con respecto a las raíces control. Las categorías más abundantes de los genes inducidos fueron la de estrés/defensa/metabolismo secundario y la de regulación/transducción de señales, mientras que la de los genes reprimidos fueron las de metabolismo de aminoácidos/proteínas y transporte/proteínas de membrana. Entre los genes que indujeron su expresión se encuentran fosfatasas, Ca^{2+}ATPasa, aldehido-deshidrogenasa, ACC oxidasa y distintos genes asociados a patogénesis y estrés oxidativo. Es probable que estos genes jueguen un papel relevante en la adaptación del frijol a la deficiencia de P, puesto que pudieran funcionar en la re-movilización y transporte de P, así como en la modificación de la arquitectura radicular y en la defensa a daño oxidativo.

Se implementó una plataforma basada en RT-PCR cuantitativo (en tiempo real) para determinar el perfil de expresión de 372 genes de FT de frijol. Estos genes de FT se identificaron con base en un análisis de dominios InterPro de las secuencias de frijol depositadas en el Bean Gene Index (Danna-Farber Cancer Institute [DFCI] Version 1). Sin embargo, este es un conjunto parcial en tanto no se conozca la secuencia completa del genoma de frijol. El análisis de la expresión de los 372 FT reveló que 17 de ellos respondieron a la deficiencia de P, cuatro de ellos fueron inducidos de 2-3.19 veces, mientras que el resto (13) fueron reprimidos de 2 – 3.03 bajo estas condiciones. Entre los FT que respondieron a la deficiencia de P, figuran tres miembros de la familia MYB y un miembro de la familia bHLH. Interesantemente, uno de los FT tipo MYB presentó una homología de 60% con AtPHR1, lo cual sugería que la vía mediada por PHR1 podía estar operando en raíces deficientes de P de frijol.

Se realizó un análisis de metaboloma basado en cromatografía de gases acoplado a espectrofotometría de masas para investigar como se afecta el metabolismo general por los cambios en la expresión génica en respuesta a –P. El análisis de metabolitos reveló que diversos azúcares mostraron un incremento en raíces deficientes de P, mientras que se observó un decremento en diversos ácidos orgánicos. La disminución en la concentración de los ácidos orgánicos (oxalacetato, malato y citrato) en raíces deficientes de P pudo deberse a que fueron exudados a la rizosfera; mientras que la acumulación de azúcares en raíces pudo ser el producto de un reajuste metabólico ocasionado por este estrés. Al respecto, se ha planteado la hipótesis de que la

acumulación de azúcres puede ser una señal que desencadena la modificación de la arquitectura radicular (Lopez-Bucio *et al.*, 2003; Vance, 2008)

En general, este trabajo aportó un número considerable de genes de frijol y metabolitos que modifican su expresión y concentración ante la deficiencia de P, respectivamente. Estos resultados pueden contribuir al entendimiento de la adaptación del frijol a la deficiencia de fósforo en condiciones no simbióticas. Además, algunos de los genes identificados en este trabajo puden ser candidatos para el fitomejoramiento molecular de esta leguminosa.

III.1.3 Análisis transcripcional de las respuestas de nódulos de frijol a la deficiencia de fósforo

Los resultados del análisis transcripcional y metabólico de las respuestas de nódulos de plantas de frijol deficientes de fósforo, se encuentran detallados en el Anexo VII.3: "Global changes in the transcript and metabolic profiles during symbiotic nitrogen fixation in phosphorus-stressed common bean plants", por: Georgina Hernández, Oswaldo Valdés-López, Mario Ramírez, Nicolas Goffard, *et al*., *Plant Physiology* DOI:10.1104/pp.109.143842.

En este trabajo se analizaron las respuestas transcripcionales de nódulos de plantas de frijol inoculadas con *Rhizobium tropici* y crecidas en deficiencia de P durante 21 días. Previo al análisis transcripcional y metabólico, se evaluaron algunos parámetros fisiológicos para determinar si nuestras condiciones experimentales inducían respuestas morfológicas y fisiológicas características de la deficiencia de P. Las plantas de frijol inoculadas y sometidas a deficiencia de P, mostraron una reducción en el contenido de este elemento, así como en el número de nódulos y en la actividad de la nitrogenasa. Lo anterior indica que la deficiencia de P afectó negativamente la formación de nódulos y la FSN. Además, estas plantas, a diferencia de plantas de frijol no simbióticas, no mostraron cambios significativos en la fotosíntesis neta, probablemente porque en estas condiciones nutricionales los nódulos aún demandan fotosintatos para el mantenimiento de los bacteroides. En conjunto, estos resultados indicaron que las plantas simbióticas de frijol presentaban respuestas características a este estrés nutricional.

El análisis transcripcional, a través de la hibridación de macro-arreglos impresos con EST provenientes de raíces deficientes de P o de nódulos control (óptimas condiciones nutricionales) de frijol, reveló que 448 genes mostraron expresión diferencial a este estrés nutricional. De estos genes, 264 (59%) indujeron su expresión, mientras que 184 genes (41 %) se reprimieron con respecto al control. Asimismo, el análisis de expresión de 372 FT por qRT-PCR indicó que 37 FT respondieron a la deficiencia de P, 36 de ellos inducidos entre 2- 6.83 veces con respecto al control. Entre los FT que se indujeron se encuentra un homólogo de AtZAT6, varios miembros de la familia AP2/EREBP (involucrados en nodulación y formación de raíces laterales), NAC, GRAS y MYB. Uno de los FT con un patrón interesante de expresión fue el regulador transcripcional TIFY, puesto que fue el único en inducir su expresión tanto en raíces como en nódulos de frijol deficientes de P.

El análisis de metaboloma reveló que en los nódulos deficientes de P, contrario a lo observado en raíces deficientes de P, se acumularon diferentes ácidos orgánicos, mientras que la acumulación de distintos azúcares y aminoácidos se observó disminuída.

Con el propósito de identificar las rutas metabólicas que fueron afectadas en nódulos deficientes de P, los datos obtenidos del perfil transcripcional fueron analizados con el software PathExpress adaptado para frijol (Goffard *et al*., 2009). Este análisis

bioinformático reveló que entre las rutas metabólicas inducidas significativamente destacan la de glicólisis y metabolismo de glicerolípidos; mientras que la síntesis de almidón se reprimió en nódulos deficientes de P. Estos datos fueron corroborados al analizar la expresión por qRT-PCR de algunos de los genes involucrados en éstas rutas metabólicas. Con este análisis se observaron correlaciones interesantes entre la inducción o represión de genes de enzimas que participan en rutas metabólicas y la modulación de la concentración de metabolitos de las vías que se modificaron en nódulos deficientes de P.

Los datos obtenidos en este trabajo, en combinación con el análisis de raíces deficientes de P, sugiere que las raíces y nódulos de plantas de frijol pueden tener un programa diferente de transcripción y de metablismo para hacer frente a la deficiencia de P. El análisis sugiere que las principales modificaciones de nódulos deficientes de P están orientadas a modificar el flujo de carbono/nitrógeno y mantener el aporte de P a través de reservas internas.

III.1.4 ¿Qué sabemos de la regulación transcripcional de las respuestas de leguminosas a la deficiencia de fósforo?

En el trabajo: "Transcriptional regulation and signaling in phosphorus starvation: What about legumes?", por: Oswaldo Valdés-López y Georgina Hernández, publicado en *Journal of Integrative Plant Biology* 50: 1213-1222 (2008) (Anexo VII.4), se hizo la integración de la información sobre los perfiles de transcripción que presentan las plantas ante la deficiencia de P y su regulación a nivel molecular. Se analizó comparativamente la información reportada para *Arabidopsis* (sistema modelo) con lo que se conoce, aún incipiente, para las leguminosas.

Se analizaron los resultados del transcriptoma y la genética directa/reversa en *Arabidopsis* que fundamentan la propuesta de que en deficiencia de P hay dos programas de transcripción: un programa "temprano" y otro "tardío". Por varios años se aceptó que el programa temprano de transcripción estaba asociado a respuestas inespecíficas, mientras que en el tardío se expresaban todos los genes asociados a la deficiencia de P. Al respecto, varios reportes indican que los genes involucrados en las adaptaciones morfológicas y bioquímicas modifican su expresión en cuestión de horas o minutos después de haber iniciado los tratamientos de deficiencia de P. Sin embargo, en los dos programas transcripcionales se han documentado la presencia de otras vías de regulación transcripcional involucradas en otros estreses, tanto bióticos como abióticos. Por lo anterior, sugerimos que existe un entrecruzamiento de rutas de regulación transcripcional, lo cual puede ser vital para la sobrevivencia de la planta.

De nuestro análisis de los datos disponibles en leguminosas, proponemos que también existen dos programas transcripcionales en frijol. Sin embargo, a diferencia de *Arabidopsis*, estos dos programas pueden ser divididos en: a) respuestas para ajustar el metabolismo de carbono/nitrógeno y adquirir P de reservas internas, y b) respuestas para modificar la morfología, para mantener la homeostasis y para adquirir P de la rizosfera. En esta parte de la revisión, se discutió el hecho de que en *L. albus*, a pesar de que se han analizado las respuestas moleculares a distintos tiempos, solo predomina un programa transcripcional: el asociado a la modificación radicular y a la adquisición de P de la rhizósfera. Quizá estos aspectos sean los que hagan diferente las respuestas de leguminosas con respecto a Arabidopsis.

Por otro lado, en este trabajo se analizaron los avances que se han hecho en *Arabidopsis* en el campo de la regulación transcripcional y post/transcripcional de las respuestas a la deficiencia de P. En esta planta modelo (hasta el 2008) se habían trazado

cuatro (actualmente 5) rutas de señalización vinculadas a 4 FT y dos riboreguladores: miR399 e *IPS1*. En este punto mencionamos que los análisis de perfiles de transcripción en frijol sugieren que algunas de estas vías de señalización pueden operar en leguminosas, pero además remarcamos algunas vías que pudieran ser específicas para estas plantas, como la vía regulada por el FT de la familia TIFY, la cual no ha sido asociada con la deficiencia de P en Arabidopsis.

Esta revisión concluye con perspectivas que hacen énfasis en la necesidad de implementar distintos enfoques genómicos que nos permitan trazar las rutas de señalización que regulan las respuestas de frijol a la deficiencia de P.

III.1. 5. La participación de PvPHR1 y PvmiR399 en la regulación de las respuestas de frijol a la deficiencia de P.

La descripción de la vía de regulación mediada por PvPHR1 y PvmiR399 se detalla en el Anexo VII. 5: "Essential role of MYB transcription factor: PvPHR1 and microRNA: PvmiR399 in phosphorus-deficiency signaling in common bean roots", por: Oswaldo Valdés-López, Catalina Arenas-Huertero, *et al*., publicado en: *Plant Cell and Environment*, 31: 1834-1843 (2008).

En este trabajó se identificaron y analizaron molecularmente los ortólogos de AtPHR1 y AtmiR399 en frijol. El análisis de la secuencia de PvPHR1 reveló la presencia de los dominios MYB y coiled-coil, además, distinto a AtPHR1, esta secuencia mostró 3 probables sitios de unión a SUMO 1, lo cual podría mediar la localización nuclear de PvPHR1. PvPHR1 se induce 1.7 veces en hojas y raíces de plantas de frijol deficientes de P.

Con el propósito de demostrar que PvPHR1 regula la expresión de posibles genes blanco que responden a la deficiencia de P, se hizo una construcción para silenciar a este gene por RNAi en plantas compuestas de frijol. Las plantas compuestas de frijol tienen raíces transgénicas, producto de la transformación genética mediada por *Agrobacterium rhizogenes* y la parte aérea no transformada. Este sistema ha resultado una alternativa eficiente para la genética reversa de frijol que es recalcitrante a la transformación genética estable (Estrada-Navarrete *et al*., 2007). En este trabajo publicamos por primera vez para frijol el silenciamiento génico por RNAi usando el sitema de raíces compuestas.

Puesto que cada planta compuesta resulta de un evento de transformación independiente, fue necesario determinar el porcentaje de silenciamiento, el cual fue diferente en cada planta. Se analizaron solamente las plantas compuestas que tuvieron un porcentaje de silenciamiento entre 80-95%. Las plantas de frijol PvPHR1-RNAi (con 90% de silenciamiento), mostraron una disminución de 60 y 30% en el contenido de P en tallos y raíces deficientes de P, respectivamente. El análisis de la expresión de 10 genes blanco que responden a la deficiencia de P, tales como *PvAPC5*, *PvAP*, *PvPht1*, *Pv4*, y el microRNA pvu-miR399, reveló que este FT regula la expresión de genes que pueden participar en la adaptación a este estrés. Se encontraron dos diferencias con respecto a lo reportado para *Arabidopsis*: la primera es que PvPHR1 regula la expresión de un gene de aquaporina, pero no la de *PvPHO1*. La segunda diferencia es que en mutantes de *Arabidopsis Atphr1*, la expresión de *APC5* (acid purple phosphatase), *AT4* (riborregulador), *RNS* (RNAasa) y *Pht1* (phosphate hight affinity transport) son reguladas parcialmente (~ 50%), lo cual ha sido explicado por el efecto sinérgico del FT AtWRKY75. En frijol estos tres genes disminuyeron su expresión en más de 90 % en plantas compuestas PvPHR1-RNAi, lo cual sugiere que la vía de PHR1 puede ser la principal vía que controla su expresión. Sin embargo, para comprobar esta

propuesta se requerirá el análisis del homólogo de frijol para AtWRKY75 y de otros FT que responden a la deficiencia de P en frijol.

Dado que en deficiencia de P, la expresión de pvu-miR399 disminuyó ~ 60 % y que los niveles de transcritos de *PvPHO2* (*Ubiquitin Conjugase: UBC24*), probable regulador negativo de *PvPht1*, se incrementó en casi 100% en plantas PvPHR1-RNAi con respecto a las plantas control, planteamos que pvu-miR399 es el regulador negativo de *PvPHO2*. Para comprobar ésta hipótesis, se procedió a disminuir la acumulación de pvu-miR399 a través del silenciamiento de *PvDCL1*. Para tal efecto, identificamos una EST con homología a *AtDCL1* y se procedió a silenciar por RNAi su expresión en frijol. Previamente se detectó que la expresión de *PvDCL1* no se modificó en plantas silvestres y PvPHR1-RNAi, tanto en condiciones control como en deficiencia de P, lo cual permitía suponer que cualquier cambio observado en la expresión de pvu-miR399, y otros miRNAs, era independiente de la deficiencia de P y PvPHR1. El análisis de plantas compuestas de frijol PvDCL1-RNAi deficientes de fósforo reveló el mismo comportamiento de pvu-miR399 y *PvPHO2* observado en las plantas PvPHR1-RNAi, lo cual sugería que pvu-miR399 es el regulador negativo de *PvPHO2*. De forma interesante, el nivel de transcritos de las fosfatasas ácidas *PvAPC5* y *PvAP* disminuyó en un 90 y 20 %, respectivamente. Estas dos fosfatasas son reguladas positivamente por PvPHR1, sin embargo, su disminución no fue dependiente de PvPHR1, puesto que en plantas compuestas PvDCL1-RNAi la expresión de este FT no fue afectada. Lo anterior sugiere que otros miRNAs u otro blanco aún no caracterizado de PvPHO2 pueden estar involucrados en su regulación. Los resultados obtenidos en este trabajo permitieron trazar una ruta de señalización de las respuestas de frijol a la deficiencia de P.

III.1.6 Identificación de microRNAs que responden al estrés nutricional en frijol

La identificación de microRNAs que responden a la deficiencia nutricional y a la toxicidad por Mn^{+2} en frijol se encuentra detallada en el Anexo VII.6: “MicroRNAs expression profile in *Phaseolus vulgaris* bean during nutrient deficiency stresses and manganese toxicity”, por: Oswaldo Valdés-López, Peter H Graham, José L Reyes, Carroll P Vance and Georgina Hernandez, enviado a revisión para su publicación en la revista *New Phytologist*, (2009).

Para cumplir el objetivo de este trabajo, se crecieron plantas de frijol en un sistema de hidroponia y fueron estresadas por –P, -Fe, -N, pH ácido (pH 5.5) y toxicidad por Mn (++Mn) durante 7 días. Estos experimentos fueron realizados en condiciones no simbióticas y simbióticas con *Rhizobium tropici* CIAT899. Bajo estas condiciones experimentales se registró una reducción entre 3 a 4 veces en los niveles de P, Fe^{+2} y N de plantas deficientes de P, Fe^{+2} y N, respectivamente, mientras que las plantas tratadas con toxicidad por Mn^{+2} mostraron un incremento drástico en el contenido de este ión. En las plantas tratadas con pH5.5 se observó una disminución de 2 veces en los niveles de Mn^{+2}, Cu^{+2} y Ca^{+2}. Asimismo, registramos una disminución en la actividad de la nitrogensa de las plantas tratadas con los diferentes estreses nutricionales. Estos datos demostraron que nuestro sistema experimental inducía respuestas características a cada estrés nutricional analizado, por lo que estas plantas se utilizaron para analizar la expresión de miRNAs.

Para poder identificar los miRNAs que responden a estos estreses nutricionales, se imprimieron macroarreglos con 70 DNA-oligonucleótidos sintéticos (denominaron miRNAs-macroarreglos) correspondientes a la secuencia complementaria-reversa del mismo número de miRNAs maduros. De estos, 24 corresponden a miRNAs conservados en distintas especies vegetales, y el resto corresponde a miRNAs

específicos de soya (35) y frijol (11) (Subramanian *et al*., 2008; Arenas-Huertero *et al*., 2009). Los miRNA-macroarreglos fueron hibridizados con una población de RNAs pequeños (marcados radiactivamente con $\gamma[^{32}P]$-ATP) extraídos de hojas, raíces y nódulos de plantas de frijol control o estresadas con –P, -Fe, -N, pH 5.5 o ++Mn.

El análisis de los perfiles de expresión de los miRNAs reveló que 32 miRNAs fueron expresados en hojas, raíces y nódulos de plantas control (concentraciones óptimas de nutrientes), 19 de ellos fueron expresados en los tres tejidos, mientras 3 de ellos sólo se expresaron en hojas y raíces, dos en hojas y nódulos, siete de ellos fueron específicos de hoja y uno fue específico de nódulo. De estos 32 miRNAs, solo 25 mostraron una expresión diferencial en los tres órganos. Algunos ejemplos de estos miRNAs son miR396, pvu-miR2118, pvu-miRS1 y pvu-miR1511, los cuales mostraron una inducción preferencial en raíces con respecto a los otros dos órganos.

El análisis del perfil de expresión de cada uno de los tres órganos de las plantas estresadas reveló que 35 miRNAs respondieron diferencialmente a los distintos tratamientos. Estos miRNAs mostraron diferentes patrones de expresión en los tres órganos, aún en un mismo tratamiento, lo cual sugería que cada órgano puede tener un programa de regulación post-transcripcional propio. En este análisis pudimos detectar miRNAs comunes entre los distintos estreses (16 miRNAs comunes) y miRNAs específicos para cada estrés, por ejemplo, detectamos que tres miRNAs (pvu-miR1514a, gma-miR1515 y gma-miR1516) fueron específicos a –Fe y sólo gma-miR1511 fue específico a pH5.5. Además de miR399, identificamos nueve miRNAs que responden a la deficiencia de P en frijol, los cuales no habían sido identificados en otras especies vegetales, incluyendo *Arabidopsis*.

Los resultados de expresión diferencial obtenidos en los miRNAs-macroarreglos, fueron validados en un 90% por experimentos tipos Northern-Blot. Asimismo, pudimos demostrar la correlación negativa entre la expresión del miR156 y de su mRNA blanco (*PvSPL6*).

Lo más interesante de este trabajo fue que varios de los miRNAs que respondieron a estos estreses nutricionales solo habían sido vinculados con procesos de desarrollo. Lo anterior permite especular que estos miRNAs y sus respectivos mRNAs blancos puedan tener varias funciones fisiológicas, o que los miRNAs tengan más mRNAs blancos de los descritos hasta el momento. Lo anterior puede ser posible, puesto que varios algoritmos pueden obviar aquellos mRNAs que no cumplan las reglas de apareamiento miRNA::mRNA establecidas anteriormente. En general, los resultados obtenidos en este trabajo aportan nueva información sobre la regulación post-transcripcional de las respuestas de frijol al estrés nutricional.

III. 2 Efectos de la toxicidad por Mn^{+2}: Resultados y Discusión.

III.2.1 Caracterización fisiológica y bioquímica de las respuestas a la toxicidad por Mn^{+2}

El manganeso (Mn^{+2}) es un micro-nutriente de vital importancia para el óptimo desarrollo de la planta puesto que participa en diversos procesos bioquímicos (Pittman, 2005). En condiciones normales, las concentraciones de Mn^{+2} en el suelo no sobrepasan el rango de 5-10 μM (Yang *et al*., 2009). Sin embargo, en algunas zonas donde se produce frijol, los suelos se caracterizan por su acidez y baja capacidad de drenaje, lo cual favorece que la disponibilidad de este nutriente sea mayor a la que normalmente requiere la planta para su desarrollo (Yang *et al*., 2009). La toxicidad por Mn^{+2} afecta negativamente la fotosíntesis, la respiración, la homeostasis de Cu^{+2}, Fe^{+2} y Ca^{+2}, estos

dos últimos nutrientes de vital importancia en la simbiosis entre rhizobia y leguminosas. Asimismo, se ha reportado que la toxicidad por Mn^{+2} estimula la producción de especies reactivas de oxígeno (ROS) y provoca estrés oxidativo (González & Lynch, 1997; González *et al*., 1998; Hall, 2002). La mayoría de estos efectos y las respuestas a la toxicidad por Mn^{+2} se han descrito en plantas que crecen en condiciones no simbióticas. A pesar de la importancia de la FSN, poco se han estudiado los efectos de la toxicidad por Mn^{+2} en los nódulos de leguminosas. Con el propósito de obtener información acerca de las respuestas de los nódulos de frijol a la toxicidad por Mn^{+2}, analizamos plantas de frijol, inoculadas con *R. tropici* CIAT 899, que fueron estresadas durante 15 días con 0.05 a 2 mM de $MnCl_2$. En comparación con nódulos de plantas control, en los nódulos de las plantas estresadas se registró un incremento de más 100 veces en el contenido de Mn^{+2}, mientras que el contenido de Fe^{+2} disminuyó en la misma magnitud (Figura 8). Una de las respuestas características en la toxicidad por Mn^{+2} es la formación de puntos cafés en las hojas, debido a la acumulación de fenoles y Mn^{+2} oxidados en la pared celular (Wisseimer & Horst, 1992). En este trabajo se observó un comportamiento similar en la superficie de los nódulos (Figura 9), lo cual no había sido documentado. Además, en los nódulos de las plantas estresadas, el número de células infectadas por CIAT899 disminuyeron considerablemente con respecto al control (Figura 10). En conjunto, estos datos indican que la toxicidad por Mn^{+2} tiene un impacto negativo en la FSN, puesto que los nódulos mostraron disminución en las células infectadas y en el contenido de Fe^{+2}, cofactor importante de la enzima nitrogenasa.

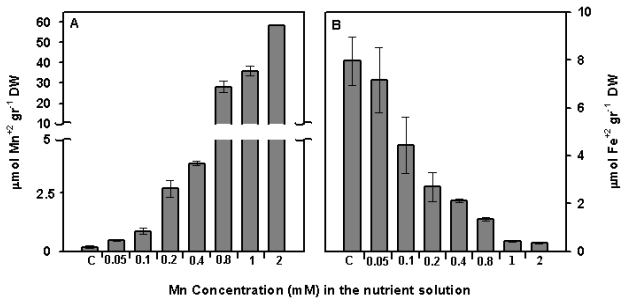


Figura 8. Concentración de Mn^{+2} (A) y Fe^{+2} (B) en los nódulos de plantas de frijol crecidas en diferentes concentraciones de $MnCl_2$. Los datos presentados son la media y el error estándar (SE) de 3 réplicas biológicas.

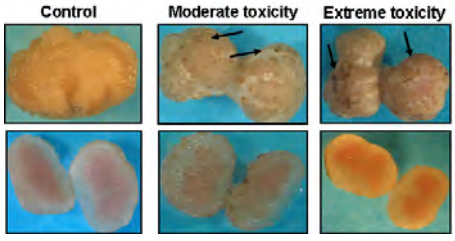


Figura 9. Síntomas visibles de la toxicidad por Mn^{+2} en nódulos de plantas de frijol crecidas en condiciones de toxicidad moderada o extrema de este ión con diferentes concentraciones de $MnCl_2$ (Ver Materales y Métodos. Anexo VII.7). Los cortes en fresco (paneles inferiores) muestran la zona de infección. Las flechas indican la formación de puntos necróticos.

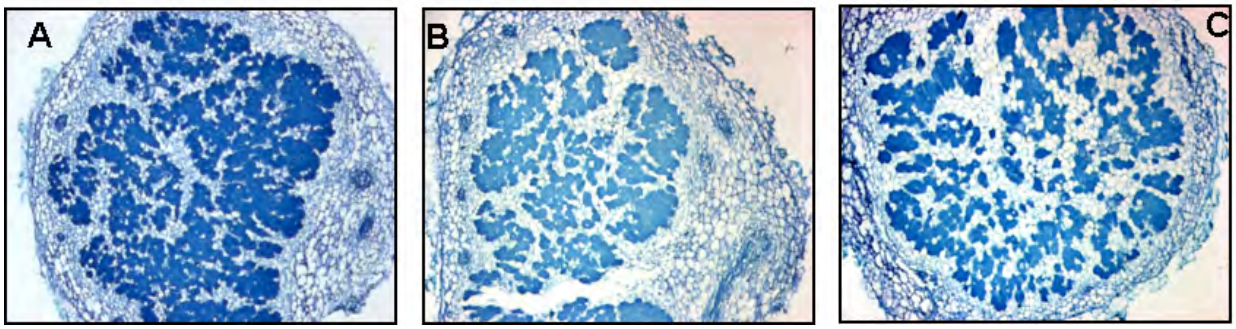


Figura 10. Histologia de nódulos de plantas de frijol crecidas en condiciones control (A), toxicidad media (B) o extrema (C) de Mn^{+2}.

Con el propósito de demostrar que la toxicidad por Mn^{+2} afecta la FSN en nódulos de frijol común, evaluamos la actividad de la enzima nitrogenasa presente en los nódulos de plantas estresadas con diferentes concentraciones de Mn^{+2} (Figura 11). Este análisis mostró que la actividad de esta enzima sólo es afectada en nódulos tratados con altas concentraciones de Mn^{+2} (0.8-2mM), puesto que en bajas concentraciones (0.05-0.4 mM) no se detecta ninguna diferencia significativa con respecto al control (Figura 11A). Puesto que la nitrogenasa tiene como cofactor el ión Fe^{+2} y en este estudio se registró una disminución de Fe^{+2} en los nódulos estresados, analizamos si existía alguna correlación entre los niveles de Fe^{+2} y la actividad nitrogenasa detectados en nódulos control y estresados (Figura 11B). Este tipo de análisis indicó que la disminución de los contenidos de Fe^{+2} y de la actividad de la nitrogenasa dependió de la cantidad de Mn^{+2} acumulado en los nódulos (Figura 8B y 11B). Además, se observó que la disminución en la actividad de la nitrogenasa está correlacionada positivamente con la cantidad de Fe^{+2} y correlacionada negativamente con la cantidad de Mn^{+2} acumulado en los nódulos (Figura 11). Estos datos demuestran que la toxicidad de Mn^{+2} afecta negativamente la FSN, probablemente a través de la disminución de los contenidos de Fe^{+2} y de los complejos Fe-Mo, esenciales para la función de la nitrogenasa en los bacteroides.

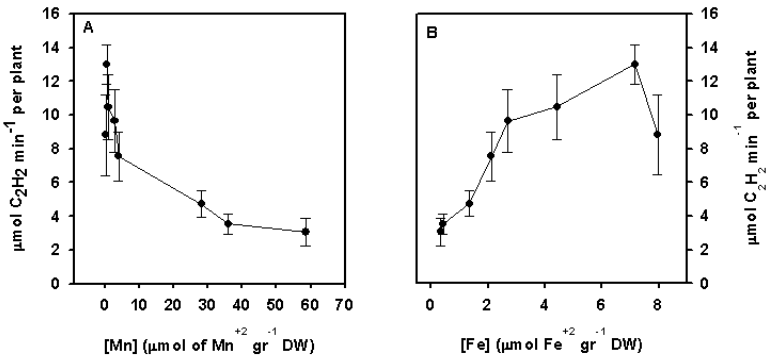


Figura 11: Determinación de la actividad de la nitrogenasa en nódulos de plantas de frijol tratadas con diferentes concentraciones de Mn^{+2}. (A) Correlación entre las cantidades acumuladas de Mn^{+2} y la actividad de la nitrogenasa. (B) Correlación entre las cantidades acumuladas de Fe^{+2} y la actividad de nitrogenasa (C). Los datos representan el promedio y el SE de 10 réplicas biológicas.

La toxicidad por Mn^{+2} en diversas especies vegetales, incluyendo leguminosas, induce la producción de especies reactivas de oxigeno (ROS), así como la modificación de la actividad de algunas enzimas antioxidativas, como peroxidasas (PX), catalasas (CAT) y super-óxido dismutasas (SOD) (González *et al*., 1998). La información acerca de la participación de enzimas antioxidativas en los nódulos de frijol estresados por Mn^{+2} es limitada. Con el propósito de identificar las enzimas antioxidativas que responden a la toxicidad por Mn^{+2} en nódulos, determinamos la actividad de la guaiacol peroxidasa (GPX), PX y de la CAT en nódulos de plantas sometidas a toxicidad moderada (0.2 mM) y extrema (1mM) de Mn^{+2}. Con respecto a las actividades enzimáticas presentes en los nódulos control, las actividades de la CAT y SOD se incrementaron 1.5 y 3 veces, respectivamente en nódulos de plantas crecidas en toxicidad extrema por Mn^{+2}. En los nódulos de plantas crecidas en toxicidad moderada, la actividad de estas dos enzimas se disminuyó 5 (SOD) y 1.3 (Catalasa) veces con respecto al control (Figura 12 A y B). Contrario a lo reportado en *Vigna unguiculata*, en donde el incremento de la actividad de la GPX es conciderado como indicador de toxicidad por Mn^{+2} en hojas de esta planta (Fecht-Christoffers *et al*., 2003), en este trabajo no se registraron diferencias significativas en los dos tratamientos de Mn^{+2} (Figura 12 C). Estos datos indican que CAT y SOD responden a la toxicidad de Mn^{+2} en nódulos de frijol, mientras que la GPX no puede ser considerada como marcador en nódulos.

III.2.2 Respuestas transcripcionales de nódulos de frijol a la toxicidad por Mn^{+2}

En estudios sobre los efectos de distintos estreses abióticos, incluyendo toxicidad por metales pesados, se ha demostrado que las respuestas a estos estímulos son reguladas a nivel transcripcional, post-transcripcional y/o trasduccional (Hall, 2002; Curie & Briat, 2003; Valdés-López & Hernández, 2008). En la toxicidad por Mn^{+2}, se han identificado distintos transportadores de Mn^{+2} involucrados en la homeostasis de este nutriente (Pittman, 2005; Delhaize *et al*., 2007; Peiter *et al*., 2007). Sin embargo, la información sobre las respuestas a este estrés a nivel transcripcional es escasa. Hasta el momento solo se ha reportado un análisis parcial del transcriptoma de hojas de plantas de *Vigna unguiculata* tratadas con 50 μM de Mn^{+2} (Führs *et al*., 2008). En el análisis se detectó la disminución de transcritos de genes asociados a fotosíntesis y respiración (Führs *et al*., 2008). Con el propósito de identificar los genes que responden a la toxicidad por Mn^{+2} en los nódulos de plantas de frijol, en este trabajo se analizó su perfil transcripcional mediante la hibridación de macroarreglos impresos con 7, 200 EST, correspondiente a 1, 786 genes únicos de nódulos de frijol. Para este análisis, se aisló RNA total de nódulos de plantas de frijol sometidas a toxicidad moderada o extrema por Mn^{+2}. Se observó que 139 genes respondieron diferencialmente a la toxicidad moderada por Mn^{+2}, de los cuales 37 fueron inducidos (Tabla 1) y 102 reprimidos (Tabla 2) más de 2 veces con respecto al control. En los nódulos de plantas de frijol sometidas a toxicidad extrema de Mn^{+2} se observó que 56 genes modificaron significativamente su expresión, 29 de ellos se indujeron (Tabla 3), mientras que 27 se reprimieron (Tabla 4).

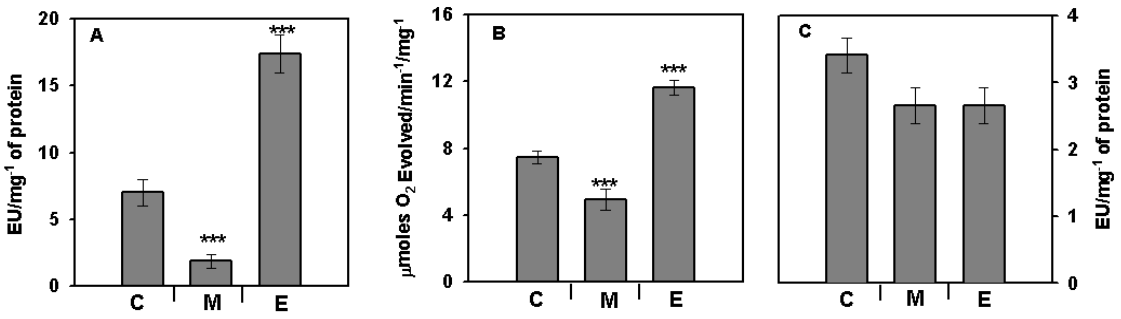


Figura 12: Determinación de la actividad enzimática de SOD (A) CAT (B) y GPX (C) en nódulos de plantas de frijol sometidas a toxicidad media (M) o extrema (E) de Mn^{+2}. Los datos representan la media y el SE de 3 réplicas biológicas. Los asteriscos sobre los histogramas indican diferencia significativa P≤0.05 respecto al control.

Las ESTs significativamente inducidas o reprimidas en los dos niveles de toxicidad por Mn^{+2} fueron anotadas, tomando como referencia la base de datos UniProt (http://www.uniprot.org/) (Tablas 1-4). De acuerdo con su anotación, los genes fueron clasificados en diferentes categorías funcionales (Tabla 1-4). Las gráficas de la Figura 14 indican los porcentajes que corresponden a cada una de las categorías funcionales según los genes inducidos o reprimidos en cada uno de los niveles de toxicidad por Mn^{+2}. La categoría funcional mayormente representada en los genes inducidos en nódulos de plantas sometidas a toxicidad moderada de Mn^{+2} fue la de metabolismo de carbono/nitrógeno (Figura 13 y Tabla 1). Dentro de esta categoría destacan los genes *glutamina sintetasa*, *uricasa-2, sacarosa sintasa* y *gliceraldehido-3-deshidrogenasa*, los cuales son importantes para el flujo de N y carbono (C). Mientras que los genes reprimidos en este tratamiento corresponden en su mayoría a proteínas desconocidas y a regulación/transducción de señales (Figura 13A y Tabla 2). En cambio, en los nódulos de plantas sometidas a toxicidad extrema de Mn^{+2} las categorías más representadas en los genes inducidos fueron los de metabolismo de aminoácidos/proteínas y metabolismo secundario/estrés/defensa; mientras que los genes reprimidos pertenecen en su mayoría al metabolismo del carbono/nitrógeno, entre los que destacan los que codifican para la sacarosa sintasa y glutamina sintetasa (Figura 13B y Tablas 3 y 4). Adicionalmente, con el análisis del transcriptoma de los nódulos sometidos a toxicidad moderada y extrema de Mn^{+2} pudimos detectar genes que pudieran estar vinculados en la detoxificación (*SNARE-asociate Golgi protein, vacuolar sorting protein*), regulación transcripcional (*heat stress transcription factor; BEL1 transcription factor; SNF7; zinc finger, CCHC type; C2H2 zinc finger protein; bHLH transcription factor*), en la transducción de señales (*MAP3K-like protein, Calmodulin*) de Mn^{+2}, así como en la defensa del estrés oxidativo (*ascorbato peroxidasa*) que genera la toxicidad de este nutriente. En general, estos datos aportan un set de genes que pudiesen tener un papel relevante en la adaptación de los nódulos de plantas de frijol a la toxicidad por Mn^{+2}. Además, sugieren que las principales modificaciones ante este estrés son en el metabolismo de carbono y nitrógeno, lo cual está acorde a los datos obtenidos en la FSN de nódulos estresados con estas dos concentraciones de Mn^{+2}.

Con el propósito de identificar genes que responden a los dos niveles de toxicidad por Mn^{+2}, los datos del transcriptoma de nódulos (Tablas 1-4) fueron

analizados por medio de diagramas de flor (Figura 14 A). Para lo cual, se construyeron 4 matrices, 2 de ellas contenían los genes inducidos y en las 2 restantes los genes reprimidos de cada tratamiento. Este análisis reveló que la respuesta de 10 genes que codifican para: *early nodulin75* (*PvENOD75*); *glutamine synthase* (*PvGS*); *early nodulin52* (*PvENOD52*); *MtN24* (*PvMtN24*); *MAP3K-like protein* (*PvMAPK*); *citosolic ascorbate peroxidase* (*PvAPX*); *SNARE associated Golgi protein* (*PvSNR*); *RNA binding protein* (*PvRbP*); *vacuolar Sorting-protein* (*PvVPS*) fue común en ambos tratamientos de Mn^{+2} (Figura 14A). Algunos de estos genes (*PvGS, PvNOD75, PvNOD52, PvNOD30*) están asociados con el metabolismo del nitrógeno y con el mantenimiento de la función del nódulo, mientras que otros (*PvAPX* y *PvMAP3K*) se han asociado a estrés oxidativo (Yeh *et al*., 2007) y al ensamblado del sistema de transporte en el aparato de Golgi (*PvSNR*) (Collins *et al*., 2003). Algunos de los 10 genes comunes a las dos concentraciones de Mn^{+2} mostraron un mismo patrón de expresión, mientras que otros mostraron inducción de su expresión en toxicidad moderada, mientras que en toxicidad extrema de Mn^{+2} reprimían su expresión, y viceversa (Figura 14A). Estos datos sugieren que probablemente estos 10 genes pueden tener un papel en la adaptación de los nódulos de frijol a la toxicidad por Mn^{+2}, ya sea que funcionen en el estrés oxidativo que genera la toxicidad por Mn^{+2} ó en el mantenimiento de la función del nódulo bajo estas condiciones de estrés.

Para confirmar que los resultados obtenidos por medio del enfoque de macroarreglos, éstos fueron validados con el método de RT-PCR semicuantitativo (sRT-PCR). Se validó la expresión de los genes que responden comunmente a ambos tratamientos de Mn^{+2} (Figura 14B). Como se observa en la Figura 14B, a excepción de *PvENOD55* y *PvPC* en toxicidad moderada y *PvSNF7* en toxicidad extrema de Mn^{+2}, el patrón de expresión determinado por macroarreglos fue confirmado por sRT-PCR. Aunque los resultados de macroarreglos fueron validados en el 80% de los casos por sRT-PCR, con respecto a la inducción y la represión, las diferencias observadas en los valores de expresión pueden deberse a la diferente sensibilidad de los dos enfoques experimentales utilizados en este trabajo.

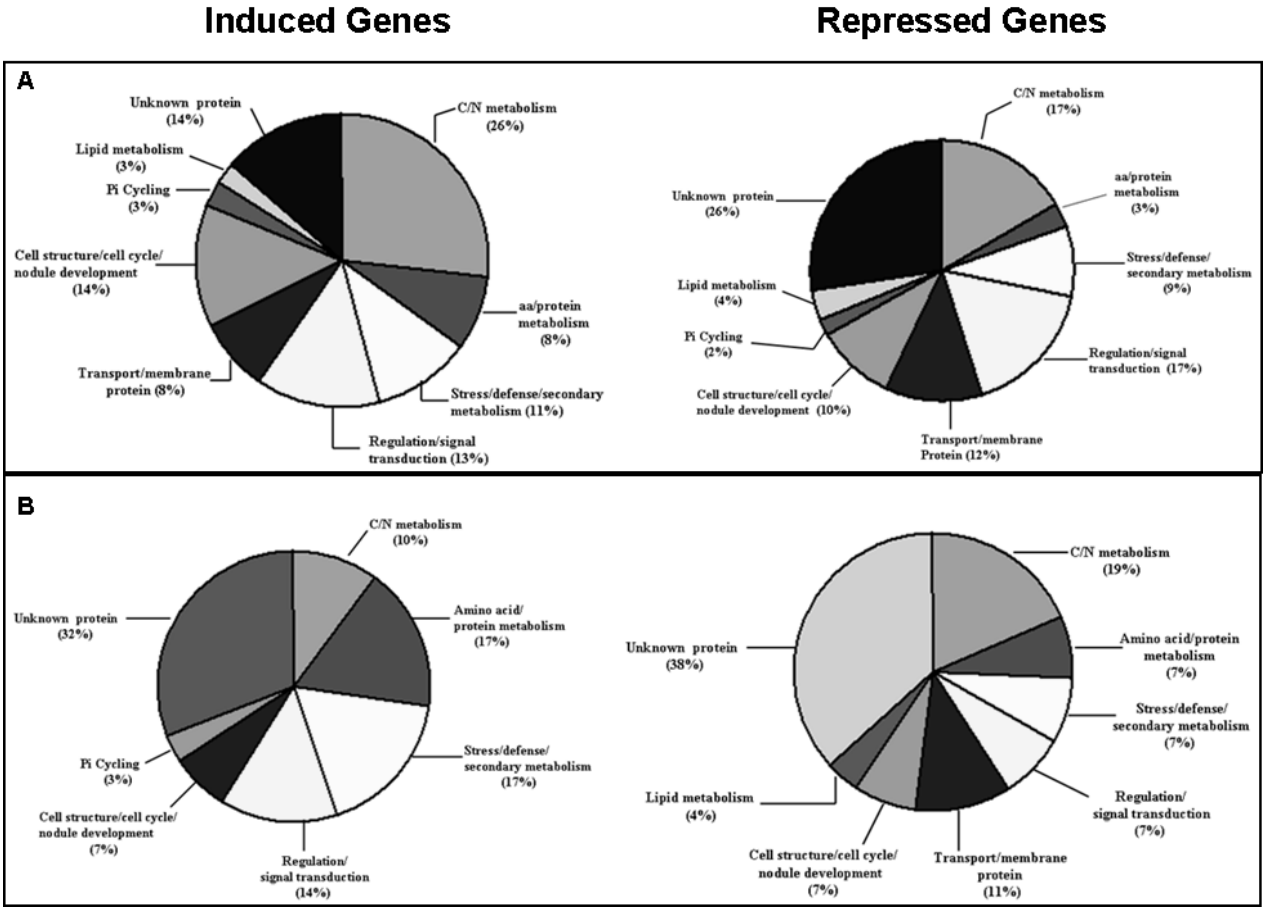
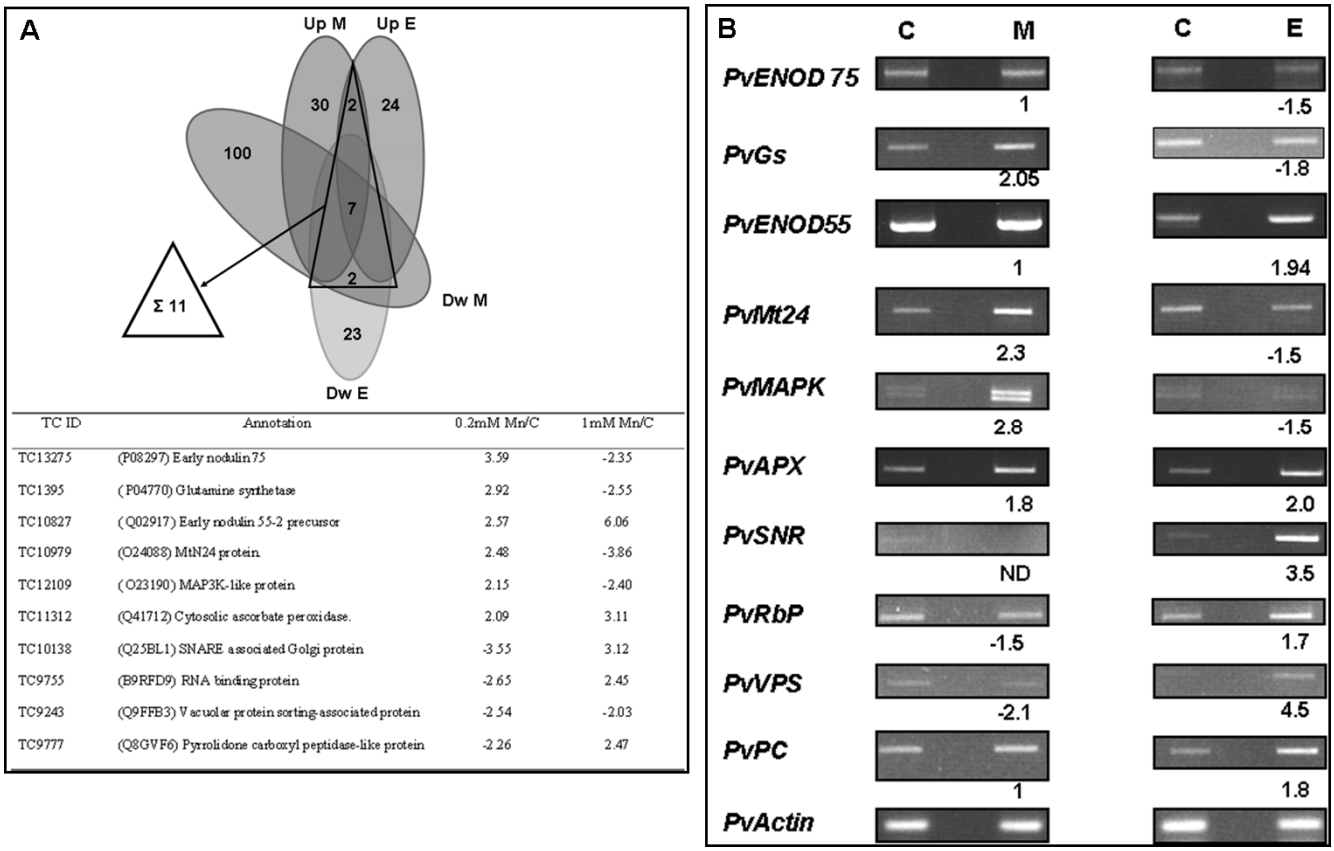


Figura 13: Distribución de los genes inducidos o reprimidos en los tratamientos de toxicidad por Mn^{+2}. Genes inducidos (derecha) o reprimidos (izquierda) más de 2 veces ($P \leq 0.05$) en nódulos de plantas tratadas con toxicidad moderada (A) o extrema (B) de Mn^{+2} con respecto a nódulos de plantas control. Los nombres de cada categoría funcional, así como el porcentaje que representa cada una, se indica en cada gráfica.



TC ID	Annotation	0.2mM Mn/C	1mM Mn/C
TC13275	(P08297) Early nodulin 75	3.59	-2.35
TC1395	(P04770) Glutamine synthetase	2.92	-2.55
TC10827	(Q02917) Early nodulin 55-2 precursor	2.57	6.06
TC10979	(O24088) MtN24 protein.	2.48	-3.86
TC12109	(O23190) MAP3K-like protein	2.15	-2.40
TC11312	(Q41712) Cytosolic ascorbate peroxidase.	2.09	3.11
TC10138	(Q25BL1) SNARE associated Golgi protein	-3.55	3.12
TC9755	(B9RFD9) RNA binding protein	-2.65	2.45
TC9243	(Q9FFB3) Vacuolar protein sorting-associated protein	-2.54	-2.03
TC9777	(Q8GVF6) Pyrrolidone carboxyl peptidase-like protein	-2.26	2.47

Figura 14. Genes de nódulos de frijol que responden a los distintos tratamientos de toxicidad por Mn^{+2}. (A) Diagrama de flor que incluye los genes inducidos (Up) o reprimidos (Dw) en condiciones de toxicidad moderada (M) o extrema (E) de Mn^{+2}. En el panel inferior se muestran los 11 genes que comunes a ambas concentraciones. (B) Validación por sRT-PCR de los resultados obtenidos de los análisis de macroarreglos. Los valores mostrados debajo de cada gene indican su nivel de expresión con respecto al control (Mn^{+2}/C). Las condiciones de PCR, así como los oligonucleotidos de cada gene se encuentran detallados en el anexo VII.8.

Recientemente se han aportado evidencias de la participación de miRNAs en la adaptación de las plantas a distintos estreses abióticos. Por ejemplo, algunos miRNAs participan en la adaptación a estrés por sequía (miR169), en la defensa contra patógenos (miR393) y en la homeostasis de P (miR399), Cu (miR398) y azufre (miR395) (Bari *et al*., 2006; Navarro *et al*., 2006; Kawashima *et al.*, 2009; Abdel-Ghany *et al.*, 2008). Además se ha reportado que miR319, miR166, y cinco miRNAs específicos de arroz (osmiR601-osmiR605) responden a la toxicidad por metales pesados (Wang *et al*., 2009). Sin embargo, la información sobre la participación de miRNAs en las respuestas a la toxicidad por Mn^{+2} es escasa. En este trabajo evaluamos la expresión de los miRNAs miR157 y miR319 en nódulos de plantas sometidas a toxicidad moderada y extrema de Mn^{+2}. Se eligieron estos dos miRNAs ya que previamente habíamos detectado su expresión en distintos órganos de plantas de frijol tratadas con 0.2 mM de Mn^{+2} en condiciones de hidroponia (Valdés-López *et al*., datos no publicados [Anexo VII.6]). Se observó que miR157 y miR319 se acumulan en los nódulos tratados con cualquiera de los dos niveles de toxicidad por Mn^{+2}; sin embargo, los nódulos de plantas sometidas a toxicidad extrema fueron los que mostraron mayor inducción (Figura 15A y B).

En distintos estudios se ha observado una correlación negativa entre la expresión de los miRNAs y sus respectivos mRNAs blancos (Abdel-Ghany & Pilon, 2008).

Recientemente, Arenas-Huertero y colaboradores (2009) reportaron los mRNAs de frijol que son blancos de varios miRNAs conservados, entre ellos los de miR157 y miR319. En este trabajo realizamos sRT-PCR con el objetivo de demostrar que los niveles de miR157 y miR319 se correlacionan negativamente con los niveles de sus mRNAs blancos: *squamosa binding protein* (*SPL6*) y *teocin protein* (*TCP*), respectivamente (Arenas-Huertero, *et al*., 2009). Comparado con los nódulos control, detectamos una disminución significativa en los niveles de *SPL6* en nódulos de plantas de frijol sometidas a los dos tratamientos de Mn^{+2} (Figura 15C), lo cual está acorde con el incremento en los niveles de miR157 (Figura 15A). En contraste, la disminución de *PvTCP* solo fue registrada en los nódulos de plantas sometidas a toxicidad extrema de Mn^{+2} (Figura 15D), en donde se registró la mayor acumulación de pvu-miR319 (Figura 15B). En conjunto, estos datos sugieren que tanto pvu-miR157 y pvu-miR319 y sus respectivos blancos pueden tener un papel fundamental en la adaptación de los nódulos de frijol a la toxicidad por Mn^{+2}. Sin embargo, no se descarta la posibilidad de que participen otros miRNAs en las respuestas de los nódulos de frijol a la toxicidad por Mn^{+2}, y de que pvu-miR157 y pvu-miR319 tengan otros mRNAs blancos, probablemente específicos para el desarrollo y mantenimiento de las funciones del nódulo.

De acuerdo a los resultados obtenidos en este trabajo, se puede concluir que la toxicidad por Mn^{+2} disminuye los niveles de Fe^{+2} de los nódulos de frijol y que probablemente este efecto contribuya en la reducción de la FSN. Además, demostramos que la toxicidad por Mn^{+2} afecta las actividades de las enzimas SOD y CAT. Asimismo, en este estudio se reportan algunos de los genes (FT, miRNAs y genes blanco) que responden diferencialmente a este estrés. Estos datos representan la base y la justificación para analizar de forma individual algunos de estos genes y de esta forma entender las adaptaciones de nódulos de frijol a la toxicidad por Mn^{+2}.

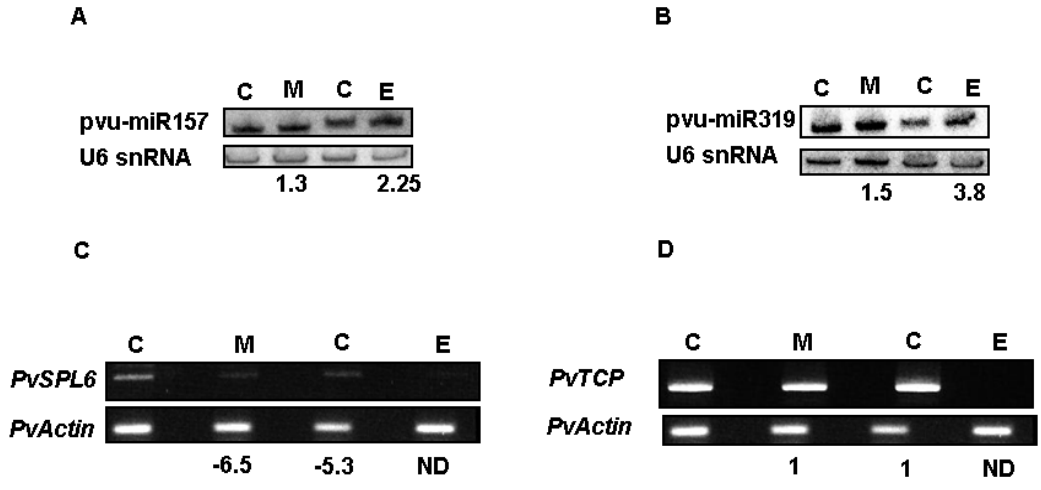


Figura 15. Determinación de la expresión de pvu-miR157 (A), pvu-miR319 (B) y de sus respectivos mRNAs blancos (C y D)en los nódulos de plantas de frijol sometidas a toxicidad moderada (M) y extrema (E) de Mn^{+2}. Los niveles de expresión de los mRNAs blancos *PvSPL6* (C) y *PvTCP* (D) se determinaron por sRT-PCR. Para cada miRNA y mRNA blanco se hicieron tres replicas biológicas. Los valores mostrados debajo de cada miRNA indican su nivel de expresión con respecto al control (Mn^{+2}/C).

Tabla 1: Genes inducidos (≥ 2, $P \leq 0.05$) en nódulos de plantas de frijol sometidas a condiciones de toxicidad moderada de Mn^{+2}.

TC/EST ID[a]	Número of EST[b]	Anotación[c]	BLASTX E-Value	Expression ratio M/C	*P-value*
Metabolismo C/N					
TC 3496	4	(Q8GTA3) Sucrose synthase	1.00E-1117	3.07	4.40E-02
TC 3396	1	(O22111) 6-phosphogluconate dehydrogenase	2.00E-67	2.98	1.23E-02
TC 4559	1	(GLNA1) Glutamine synthetase	8.00E-81	2.92	2.95E-02
TC 3014	6	(P04670) Uricase-2	1.00E-119	2.90	8.38E-04
TC 3153	2	(Q8H2A9) L-asparaginase	2.00E-56	2.77	2.39E-02
TC 7050	2	(A5B118) Fructose-bisphosphate aldolase	1.00E-77	2.67	1.62E-02
TC 4263	1	(NDUS6) NADH dehydrogenase [ubiquinone]	1.00E-35	2.31	3.97E-02
TC 3835	1	(Q38JJ4) Fructose-bisphosphate aldolase, cytoplasmic isozyme 2	2.00E-28	2.14	7.06E-04
TC 7272	1	(Q6RIB7) Enolase	1.00E-117	2.14	3.86E-03
TC 3218	1	(Q07CZ3) Glyceraldehyde-3-dehydrogenase C subunit	4.00E-89	2.09	9.87E-03
Metabolismo de Aminocidos/Proteínas					
TC 3217	2	(Q9SUU0) Serine hydroxymethyltransferase	1.00E-113	2.50	3.05E-03
TC 5370	1	(Q41713) Aspartic proteinase	1.00E-103	2.24	7.25E-03
TC 3918	2	(Q1KSI5) S-adenosyl-L-methionine synthetase	2.00E-80	2.20	1.03E-03
Etres/defensa/metabolismo secundario					
TC 3585	5	(Q6XBF8) CDR1	2.00E-39	2.61	3.21E-02
TC 5419	1	(P35055) Coproporphyrinogen III oxidase, chloroplast precursor	1.00E-100	2.22	4.29E-02
TC 3536	3	(Q41712) Cytosolic ascorbate peroxidase.	1.00E-100	2.09	1.66E-02
TC 8035	1	(A6YGE4) Hypersensitive-induced response protein.	3.00E-94	2.01	3.93E-03
Regulaciónn/transducción de señales					
TC 3358	1	(Q93XA5) Homeodomain leucine zipper protein HDZ1	8.00E-92	3.21	2.73E-02
TC 1740	1	(Q10MK9) AMP-binding enzyme family protein	6.00E-87	2.84	3.70E-03
TC 4510	1	(Q9LLF2) Heat stress transcription factor A-3	1.00E-51	2.33	2.16E-02
TC 6780	1	(Q2HVD1) IQ Calmodulin-binding region	8.00E-16	2.27	1.50E-03
TC 3953	1	(Q69U53) MAP3K-like protein	2.00E-47	2.15	2.92E-02
Transporte/proteínas de membrana					
TC 3138	1	(P2B10) F-box protein PP2-B10	2.00E-54	4.01	3.93E-02
TC 4100	1	(O80412) Mitochondrial phosphate transporter.	1.00E-103	2.07	3.21E-03
NOD_247_H02	S	(A7YGJ3) Sulfate transporter	2.00E-67	2.02	3.21E-03

Tabla 1: (Continuación de la página anterior).

TC/EST ID[a]	Número de EST[b]	Annotacion[c]	BLASTX E-Value	Expression ratio M/C	*P-value*
Est. celular/ciclo celular					
TC 4601	4	(P08297) Early nodulin 75	4.00E-50	3.59	2.42E-02
TC 4926	1	root nodule extension	¿?	3.09	2.67E-03
TC 4022	1	(Q41805) Extensin-like protein precursor. (Cambio anotacion: Phosphoribosil)	1.40E-06	3.09	3.81E-02
NOD_226_H01	S	(Q9C8A0) Serine/arginine-rich protein	5.00E-19	2.75	4.95E-03
TC 3307	1	(NO552) Early nodulin 55-2 precursor	1.00E-56	2.57	1.88E-03
TC 3458	1	(Q6T7D1) Fiber protein Fb19	1.00E-46	2.53	2.42E-02
TC 4328	1	(Q9LSF6) Nodulin 21 (N-21).	9.00E-41	2.02	2.17E-03
Reciclado de Pi					
TC 3292	1	(Q8GT38) Putative phosphatase.	1.00E-111	2.16	1.29E-02
Metabolismo de lípidos					
TC 3993	2	(Q0ZPW9) CXE carboxylesterase	5.00E-54	2.76	2.78E-03
Proteínas desconocidas					
TC 3518	1	(A5BGJ2) Putative uncharacterized protein	8.00E-27	2.76	1.79E-02
TC 3370	2	(O24088) MtN24 protein.	2.00E-30	2.48	1.54E-02
TC 6585	1	No blast hit	----------	2.08	4.38E-02

Las categorías funcionales se indican en negritas. [a]TC: Tentative Consensus sequence assignment (TIGR/DFI Common Bean Gene Index, version 2.0). [b]Numero de EST indica las ESTs de un específico TC que fue diferencialmente expresado en esta condición. “S” indica un singleton con idéntico ID. Para TC con más de una EST, los datos mostrados son el promedio. [c]La anotación funcional fue hecha al comprar todos los TC ó S con la base de datos UniProt.

Tabla 2: Genes reprimidos (≥ 2, $P \leq 0.05$) en nódulos de plantas de frijol sometidas a condiciones de toxicidad moderada de Mn^{+2}.

TC/EST ID[a]	Número de EST[b]	Anotación[c]	BLASTX E-Value	Expression ratio C/M	*P-value*
Metabolismo de C/N and S					
TC 5998	1	(OPD23) Dihydrolipoyllysine-residue acetyltransferase	2.00E-76	6.23	5.09E-02
TC 3495	1	(Q852S0) Pyruvate dehydrogenase E1alpha subunit	6.00E-80	3.59	3.53E-02
NOD_215_H03	S	(A2Q544) FAD linked oxidase, N-terminal	1.00E-122	3.43	1.36E-03
NOD_245_A01	S	(A2SXR3) Urate oxidase	1.00E-50	3.23	2.42E-02
TC 3283	1	(Q5JMX4) Putative GTPase	1.00E-79	3.06	2.59E-02
NOD_204_B03	S	(Q2V993) Sulfite oxidase-like	1.00E-153	2.63	1.83E-02
TC 7945	1	(P00052) Cytochrome c	2.00E-58	2.98	1.75E-04
NOD_245_B07	S	(Q8H103) Glucose-6-phosphate isomerase	1.00E-140	2.86	2.67E-02
TC 3426	1	(O81226) Glutamine cyclotransferase precursor	2.00E-71	2.51	5.67E-03
TC 4642	S	(Q6Z702) Putative 3-isopropylmalate dehydratase	2.00E-72	2.42	2.03E-02
NOD_201_D05	S	(Q07A04) Phosphatidylglycerolphosphate synthase	1.00E-117	2.22	3.46E-02
TC 5338	1	(KAD2) Adenylate kinase B	6.00E-60	2.15	3.12E-02
NOD_234_C07	S	(Q5I190) Beta-galactosidase	1.00E-129	2.09	1.67E-02
TC 3289	1	(Q5JZZ3) Triosephosphate isomerase	3.00E-98	2.01	3.82E-03
Metabolismo de Aminocidos/Proteínas					
TC 4577	1	(Q677H6) ADP-ribosylation factor	6.00E-96	4.81	1.37E-02
TC 3188	1	(Q8L5P9) Ubiquitin carrier protein	2.00E-89	4.27	2.68E-03
TC 4297	1	(RS191) 40S ribosomal protein S19-1	1.00E-70	2.34	2.94E-02
TC 3208	1	(Q49RB5) Ubiquitin carrier protein	7.00E-87	2.25	2.05E-02
TC 6235	1	(Q7XBI1) Metacaspase 7	6.00E-66	2.21	3.09E-02
TC 4463	1	(RK17) 50S ribosomal protein L17, chloroplast precursor (CL17)	1.00E-70	2.00	4.74E-02
Estres/defensa/metabolismo secundario					
NOD_201_D04	S	(Q4ZGK1) Beta-1,3-glucanase 1	2.00E-37	3.03	7.65E-03
NOD_208_G01	S	(Q9LKH7) Cytochrome P450	1.00E-135	2.47	3.73E-02
TC 5659	1	(UFOG5) Anthocyanidin 3-O-glucosyltransferase	2.00E-30	2.42	3.35E-02
TC 7586	1	(Q947E1) Resistance gene analog NBS7	4.00E-41	2.27	4.85E-03
TC 4834	1	(Q8GVF6) Pyrrolidone carboxyl peptidase-like protein	1.00E-85	2.26	1.64E-02
TC 4148	1	(Q38JC8) Temperature-induced lipocalin	4.00E-74	2.24	5.19E-03
TC 8073	1	(Q8LN96) Putative DnaJ domain containing protein	4.00E-56	2.17	4.35E-02
TC 3722	1	(B4UW73) Universal stress protein	1.00E-47	2.16	2.38E-02
TC 2928	1	(O24315) Cinnamate 4-hydroxylase	3.00E-95	2.14	1.58E-02

Tabla 2: (Continuación de la página anterior).

TC/EST ID[a]	Número de EST[b]	Anotación[c]	BLASTX E-Value	Expression ratio C/M	*P-value*
Regulaciónn/transducción de señales					
NOD_242_B08	S	(Q52KW8) Regulator of Chromosome condensation (RCC1)	7.00E-29	3.81	6.06E-03
TC 6782	1	(Q71R35) Translational initiation factor eIF1	1.00E-54	3.69	9.03E-03
NOD_247_A06	S	(Q2HRD6) Translation protein SH3-like	3.00E-80	3.67	1.02E-02
NOD_239_A08	S	(Q5JKM9) Transducin-like protein	6.00E-82	3.67	5.24E-03
TC 5239	1	(Q2PF41) BEL1-like homeodomain transcription factor	1.00E-117	3.14	3.38E-04
TC 6701	1	(A6MD19) EAP30	4.00E-84	3.13	1.45E-02
NOD_243_E09	S	(Q6L561) Putative nuclear RNA binding protein A	3.20E-06	2.97	3.31E-02
TC 4201	1	(Q659S8) Putative His-Asp phosphotransfer protein	2.00E-58	2.94	6.52E-03
TC 3600	1	(Q71F50) Eukaryotic translation initiation factor 5A isoform II	2.00E-85	2.77	1.16E-04
NOD_230_E01	S	(Q0WLM3) Zinc finger like protein	2.00E-55	2.75	4.03E-02
TC 7889	1	(RH30) DEAD-box ATP-dependent RNA helicase 30	1.00E-107	2.69	2.97E-02
TC 3043	1	(Q71V71) Calmodulin	3.00E-80	2.68	1.98E-02
TC 5817	1	(Q10SX2) SNF7	9.00E-27	2.54	8.39E-03
NOD_245_D03	S	(Q1SAX2) Zinc Finger, CCHC-type	6.00E-16	2.42	1.77E-03
NOD_246_A10	S	(Q6ST18) Heat shock factor binding protein 2	1.00E-20	2.40	3.51E-02
NOD_203_A03	S	(Q40581) EF-1-alpha-related GTP-binding protein	2.00E-42	2.39	2.22E-02
TC 5136	1	(Q9SAU1) TATA-box-binding protein	2.00E-91	2.21	4.62E-02
Transporte/proteínas de membrana					
TC 3671	1	(Q42477) Vacuolar ATP synthase catalytic subunit A	1.00E-95	4.49	9.01E-04
TC 3246	1	(Q6K4N7) Amino acid transporter-like	6.00E-86	3.62	2.68E-02
NOD_224_A10	S	(Q2HTA9) Ankyrin	4.00E-61	3.61	2.50E-02
TC 3103	1	(Q25BL1) SNARE associated Golgi protein	1.00E-97	3.55	1.59E-02
TC 4285	1	(A2Q4T7) Mitochondrial import inner membrane translocase	3.00E-58	3.00	2.19E-02
TC 3030	1	(Q43631) Nodulin 30	1.00E-102	2.87	9.78E-05
TC 4947	3	(NOD30_PHAVU) Nodulin 30 precursor	5.00E-55	2.75	1.36E-02
TC 5270	1	(Q2PF08) Putative ADP ATP carrier protein	8.00E-81	2.80	4.78E-02
TC 4421	1	(Q7XJQ3) Putative peptide/amino acid transporter	7.00E-44	2.52	4.46E-02
TC 3448	1	(ATP4) ATP synthase	2.00E-74	2.26	2.24E-02
TC 3676	1	(Q9ZWN0) GPI-anchored protein	5.00E-63	2.20	4.11E-02
NOD_241_F06	S	(Q9CAN1) Membrane protein	6.00E-14	2.10	4.64E-03
TC 4611	10	(Q03972) Leghemoglobin	6.00E-68	2.09	2.05E-02
TC 3449	1	(Q9AX79) Vacuolar H+-exporting ATPase	2.00E-76	2.05	4.90E-02
TC 3353	1	(A1CQ66) F1F0 ATP synthase assembly protein	8.00E-04	2.04	9.22E-03

Tabla 2: (Continuación de la página anterior).

TC/EST ID[a]	Número de EST[b]	Anotación[c]	BLASTX E-Value	Expression ratio C/M	*P-value*
Est. Celular/Ciclo celular					
NOD_241_E08	S	(SKU5) Putative monocopper oxidase precursor	6.00E-80	4.81	2.98E-02
TC 3350	1	(Q683G1) Similar to senescence-associated protein	2.00E-82	3.78	8.64E-03
TC 4940	1	(A2Q5C7) Cyclin-like F-box	2.00E-39	3.33	1.64E-02
TC 5418	1	(Q0EAF4) Serine/threonine-protein phosphatase PP1	1.00E-127	3.06	8.30E-03
TC 3270	1	(O65848) Annexin	1.00E-113	2.76	4.15E-02
TC 5709	1	(A7QSR6) Auxin Efflux Carrier	5.00E-42	2.66	1.91E-02
TC 3979	1	(Q6F4H4) Actin	0	2.58	2.71E-02
NOD_240_G10	S	(Q8RWV5) Putative cell differentiation protein	7.00E-97	2.42	3.20E-02
TC 7168	1	(Q6YBV3) Cellulose synthase	1.00E-115	2.25	9.60E-03
TC 5726	1	(Q7F1U6) Adhesive/proline-rich protein	8.00E-34	2.23	4.65E-02
TC 5870	1	(TCTP) Translationally-controlled tumor protein	4.00E-78	2.16	1.71E-02
Reciclado de Pi					
NOD_220_B05	S	(Q0DD22) Acylphosphatase	7.00E-21	2.99	1.04E-03
TC 3593	1	(Q2P9V0) Soluble inorganic pyrophosphatase	2.00E-87	2.19	2.71E-02
Metabolismo de Lípidos					
NOD_243_F10	S	(A7L830) 3-ketoacyl-CoA synthase	1.00E-59	3.53	3.35E-02
TC 3750	1	(Q9C5C4) Putative N-acetylornithine deacetylase	1.00E-91	3.33	2.72E-02
TC 3695	1	(Q8LEU1) Acyl carrier protein, putative	1.00E-40	2.74	4.60E-02
TC 3689	1	(Q8RVT5) Acyl-CoA-binding protein	9.00E-37	2.09	2.83E-03
Proteínas desconocidas					
TC 7439	1	(Q2HT92) Putative uncharacterized protein	4.00E-13	5.71	8.38E-03
NOD_203_C08	S	No Blast Hit	----------	4.68	1.78E-02
NOD_212_D06	S	No Blast Hit	----------	4.17	2.35E-02
TC 4787	1	(Q3ED57) Uncharacterized protein	3.00E-25	3.75	1.77E-02
TC 6749	1	No Blast Hit	----------	3.55	5.92E-04
TC 3268	S	(Q9SLE2) Expressed protein	3.00E-76	3.47	4.52E-02
NOD_208_A05	1	(Q8GZ36) Putative uncharacterized protein	8.00E-44	3.18	1.21E-02
TC 6866	1	(Q8GZ36) Putative uncharacterized protein	1.00E-19	3.18	2.92E-02
TC 5401	1	No Blast Hit	----------	3.15	1.04E-02
NOD_235_B10	S	(Q2HVI0) Putative uncharacterized protein	2.00E-28	3.08	3.14E-02

Tabla 2: (Continuación de la página anterior).

TC/EST ID[a]	Número de EST[b]	Anotación[c]	BLASTX E-Value	Expression ratio C/M	*P-value*
Proteínas desconocidas					
NOD_214_F07	S	No Blast Hit	----------	2.87	1.42E-02
TC 6876	1	(A7NTH0) Chromosome chr18 scaffold_1	1.00E-45	2.79	2.81E-02
NOD_243_C11	S	(A7PRQ1) Chromosome chr14 scaffold_27	1.00E-84	2.77	3.10E-03
NOD_230_C09	S	(A7QYN3) Chromosome undetermined scaffold_252	2.00E-52	2.74	8.99E-03
TC 3040	1	(A7PRY7) Chromosome chr14 scaffold_27	9.00E-13	2.65	1.64E-03
NOD_230_E11	S	(Q9S7T5) Putative uncharacterized protein	3.00E-27	2.57	5.54E-03
TC 6401	1	(Q8H6S5) CTV.2	4.00E-93	2.49	2.00E-02
TC 6277	1	(YCF68) Uncharacterized protein	3.00E-43	2.42	4.07E-02
TC 3329	1	(A7QZ06) Chromosome undetermined scaffold_261	4.00E-45	2.42	3.68E-03
NOD_225_D01	S	(A5BES1) Putative uncharacterized protein	2.00E-43	2.39	3.57E-02
TC 6939	1	(Q9SJL4) Expressed protein	1.00E-50	2.33	1.84E-04
NOD_245_C11	S	(Q0CR44) Putative uncharacterized protein	1.00E-06	2.30	1.03E-03
TC 3667	1	(A7NUZ6) Chromosome chr18 scaffold_1	5.00E-36	2.28	3.20E-02
NOD_248_G12	S	(A7P5Z2) Chromosome chr4 scaffold_6	8.00E-26	2.20	4.40E-03
TC 4468	1	(Q9C574) Putative uncharacterized protein	7.00E-95	2.19	6.61E-03
TC 5327	1	(Q8GWG9) Putative uncharacterized protein	7.00E-61	2.17	2.56E-02
TC 5860	1	(Q9AV95) Putative uncharacterized protein	3.00E-52	2.16	1.84E-02

Las categorías funcionales se indican en negritas. [a]TC: Tentative Consensus sequence assignment (TIGR/DFI Common Bean Gene Index, version 2.0). [b]Número de EST indica las ESTs de un específico TC que fue diferencialmente expresado en esta condición. “S” indica un singleton con idéntico ID. Para TC con más de una EST, los datos mostrados son el promedio. [c]La anotación funcional fue hecha al comprar todos los TC ó S con la base de datos UniProt.

Tabla 3: Genes inducidos (≥ 2, $P \leq 0.05$) en nódulos de plantas de frijol sometidas a condiciones de toxicidad extrema de Mn^{+2}.

TC/EST ID[a]	Número de EST[b]	Anotación[c]	BLASTX E-Value	Expression ratio E/C	*P-value*
Metabolismo de C/N					
NOD_202_B10	S	(Q5SNH5) Putative 6-phospho-1-fructokinase	1.00E-60	9.49	3.35E-02
TC 6955	1	(Q8S4Q4) Noduline 6 (Glutamate-ammonia ligase)	9.00E-74	3.68	4.21E-02
TC 3282	1	(Q8S9A7) Glucosyltransferase-2.	6.00E-90	2.18	1.80E-02
Metabolismo de Aminocidos/Proteínas					
TC 3392	1	(Q10MJ1) Prolyl oligopeptidase family protein	1.00E-116	3.67	3.10E-02
NOD_235_G09	S	(Q8W311) Putative RNA-binding protein	6.00E-55	2.83	4.70E-02
TC 7224	1	(Q45W77) Ubiquitin carrier protein	3.00E-83	2.48	1.93E-02
TC 4834	1	(Q8GVF6) Pyrrolidone carboxyl peptidase-like protein	4.00E-76	2.47	1.86E-03
TC 3417	1	(O48955) Putative RNA binding protein	3.00E-54	2.35	2.75E-02
Etres/defensa/metabolismo secundario					
TC 3115	1	(Q94B32) Similar to dihydroflavonol reductase	1.00E-130	4.13	3.48E-02
TC 7348	1	(Q9M4E6) Heat-shock protein	7.00E-80	4.08	2.06E-02
TC 3536	4	(Q41712) Cytosolic ascorbate peroxidase	1.00E-109	3.11	1.61E-03
TC 3276	1	(Q5MCR8) aluminum induced protein	7.00E-96	2.98	3.21E-02
TC 3157	1	(Q9ZSZ6) DnaJ protein	2.00E-65	2.90	3.43E-02
Regulación/transducción de señales					
NOD_203_D08	S	(A5JUQ4) C2H2 zinc finger protein	2.00E-31	5.89	4.23E-02
NOD_236_A04	S	(O82658) putative kinase	6.00E-55	4.30	4.83E-02
TC 3371	1	(Q9SRM) Nucleic acid binding protein	6.00E-59	3.66	5.06E-02
TC 4430	1	(Q9M2L8) Translation initiation factor 3-like protein	8.00E-78	2.95	4.10E-02
NOD_224_C12	S	(Q5JJJ5) Putative dynamin like protein 2a	3.00E-52	2.79	5.06E-02
TC 6310	1	(PA2_HIS) Phospholipase A2	8.00E-48	2.49	3.34E-02
Est. celular/ciclo celular/des. de nódulo					
TC 3307	1	early nodulin 55-2 (glycoprotein)	1.00E-56	6.06	3.42E-02
TC 4635	1	(A7KQH2) Beta-tubulin 6	5.00E-88	4.59	2.62E-02
Transporte/proteínas de membrana					
TC 3103	1	(Q25BL1) SNARE associate Golgi protein	1.00E-97	3.12	3.51E-03
Reciclado de P					
NOD_236_D10	**S**	(Q9SZ11) Probable glycerophosphoryl diester phosphodiesterase 2	2.00E-31	3.08	1.24E-02

Table 3: (Continuación de la página anterior)

TC/EST ID[a]	Número de EST[b]	Anotación[c]	BLASTX E-Value	Expression ratio E/C	*P-value*
Proteínas desconocidas					
TC 3232	1	(Q9SL69) Expressed protein	1.00E-106	6.18	3.87E-02
NOD_224_C03	S	No hits found	----------	3.42	1.41E-02
TC 4013	1	(Q9LXT4) Putative uncharacterized	1.00E-92	2.47	2.27E-02
TC 3040	1	(A9PAM8) Putative uncharacterized	8.00E-31	2.45	1.54E-02
TC 3847	1	(Q8L4Q6) Putative uncharacterized	8.00E-54	2.37	1.98E-02
TC 7451	1	(Q8RWV9) Putative uncharacterized protein	5.00E-06	2.23	1.59E-03

Las categorías funcionales se indican en negritas. [a]TC: Tentative Consensus sequence assignment (TIGR/DFI Common Bean Gene Index, version 2.0). [b]Número de EST indica las ESTs de un específico TC que fue diferencialmente expresado en esta condición. "S" indica un singleton con idéntico ID. Para TC con más de una EST, los datos mostrados son el promedio. [c]La anotación funcional fue hecha al comprar todos los TC ó S con la base de datos UniProt.

Table 4: Genes reprimidos (≥ 2, $P \leq 0.05$) en nódulos de plantas de frijol sometidas a condiciones de toxicidad extrema de Mn^{+2}.

TC/EST ID[a]	Número de EST[b]	Anotación[c]	BLASTX E-Value	Expression ratio C/E	*P-value*
Metabolismo de C/N					
TC 3159	4	(GLNA3) Glutamine synthetase N-1	1.00E-62	5.03	4.60E-03
NOD_217_C06	S	(Q8S996) Glucosyltransferase-13	8.00E-60	2.82	2.98E-02
TC 4559	2	(GLNA1) Glutamine synthetase PR-1	3.00E-96	2.55	3.33E-02
TC 4530	1	(Q8LBS7) Putative nitrilase-associated protein	7.00E-27	2.15	1.61E-02
TC 4272	1	(Q07CZ3) Glyceraldehyde-3-dehydrogenase C subunit	1.00E-120	2.01	8.40E-03
Metabolismo de Aminocidos/Proteínas					
TC 3469	5	(Q9AT56) S-adenosylmethionine synthetase	1.00E-89	2.65	4.94E-03
TC 7654	1	(Q9SHP0) Putative phosphoserine aminotransferase	5.00E-50	2.62	1.80E-02
Estres/defensa/metabolismo secundario					
TC 6667	1	(Q2HVK9) Tetrahydrofolate dehydrogenase/cyclohydrolase	1.00E-93	2.86	2.90E-02
TC 3033	1	(PHAVU) Pathogenesis-related protein 1	6.00E-84	2.07	3.03E-02

Table 4: (Continuación de la página anterior)

TC/EST ID[a]	Número de EST[b]	Anotación[c]	BLASTX E-Value	Expression ratio C/E	*P-value*
Regulación/transdución de señales					
TC 4742	1	(Q8S3F3) Putative bHLH transcription factor	1.00E-29	3.46	1.68E-02
TC 3611	1	(Q9LSQ0) AMP-binding protein	1.00E-15	2.71	3.55E-02
TC 3428	1	(Q9FVD4) Ser/Thr specific protein phosphatase 2A	1.00E-113	2.49	1.90E-02
TC 3953	1	(Q69U53) MAP3K-like protein	1.00E-85	2.40	3.80E-02
NOD_204_D04	S	(Q54KX0) GATA zinc finger	8.00E-04	2.09	1.05E-02
TC 5817	1	(Q10SX2)SNF7	9.00E-27	2.03	5.03E-02
Transporte/proteínas de membrana					
TC 5571	S	(Q8LJR4) Syntaxin	4.00E-89	3.24	3.47E-02
TC 4084	1	(Q9SUV1) Adenylate translocator	2.00E-36	2.46	3.11 E-02
TC 4890	1	(Q6U1L7) Bax inhibitor	9.00E-50	2.01	4.77E-02
Est. Celular/ciclo celular					
TC 4972	1	(Q84XA3) Inosine monophosphate dehydrogenase	2.00E-91	2.56	1.07E-02
TC 4947	14	Nodulin 30 precursor	5.00E-94	2.35	2.72E-02
TC 4601	1	(P08297) Early nodulin 75	2.00E-23	2.35	3.25E-02
Metabolismo de Lípidos					
TC 3993	1	(Q0ZPW9) CXE carboxylesterase	5.00E-54	3.83	1.31E-02
Proteínas desconocidas					
TC 3370	1	(O24088) MtN24 protein	2.00E-12	3.86	2.06E-02
TC 6565	1	(A8SBJ8) Putative uncharacterized protein	4.00E-29	3.15	1.03E.03
TC 3566	1	(Q10MR5) Expressed protein	5.00E-26	2.91	1.15E-02
TC 6336	1	No blast hit	----------	2.46	3.87E-02
TC 7566	1	(Q5Z414) Putative uncharacterized protein	2.00E-15	2.23	3.97E-02
NOD_219_E01	S	No blast hit	----------	2.14	2.90E-02
TC 5622	1	(A5BE58) Putative uncharacterized protein.	3.00E-54	2.00	5.94E-03

Las categorías funcionales se indican en negritas. [a]TC: Tentative Consensus sequence assignment (TIGR/DFI Common Bean Gene Index, version 2.0). [b]Número de EST indica las ESTs de un específico TC que fue diferencialmente expresado en esta condición. “S” indica un singleton con idéntico ID. Para TC con más de una EST, los datos mostrados son el promedio. [c]La anotación funcional fue hecha al comprar todos los TC ó S con la base de datos UniProt.

IV. DISCUSIÓN GENERAL

A continuación se presenta una discusión general de los datos obtenidos en esta tesis doctoral. Sin embargo, cada publicación contiene la discusión de los datos presentados en cada una de ellas.

El frijol es una de las principales fuentes de proteínas y minerales en la dieta diaria de pobladores de países emergentes como México. Además, el frijol, juega un papel importante en el ecosistema, puesto que a través de la simbiosis con rhizobios incorpora el nitrógeno atmosférico al metabolismo de otros organismos. Sin embargo, la calidad nutricional de los suelos en los que crece el frijol es una limitante para la producción y la calidad de la semilla de esta leguminosa, así como para la FSN (Brougthon *et al*., 2003). Existen diversos estudios en los que se reportan las respuestas morfológicas y bioquímicas del frijol al estrés nutricional (-P, -Fe o toxicidad por metales pesados) (revisado por: Valdés-López & Hernández, 2008; Vance, 2008). Sin embargo, poco se sabe al respecto de sus respuestas transcripcionales y post-transcripcionales, así como de las vías de señalización que participan en el control de las respuestas morfológicas y bioquímicas. En el presente trabajo, por medio del enfoque de macroarreglos se identificaron ~600 genes que responden a la deficiencia de P en nódulos y raíces de frijol; 195 genes que responden diferencialmente al exceso de Mn^{+2} en nódulos de frijol, y 35 miRNAs que respondieron diferencialmente a distintos estreses nutricionales. Además, por medio de los enfoques de metaboloma y bioinformáticos por Pathexpress, identificamos los metabolitos y las rutas metabólicas que se modulan en respuesta a la deficiencia de P. En conjunto, estos resultados pueden ayudar a entender a nivel molecular y bioquímico las respuestas de frijol común al estrés nutricional.

La modificación de la arquitectura radicular es una de las respuestas más características en la deficiencia nutricional (López-Bucio *et al*., 2003; Vance, 2008). Las modificaciones de la arquitectura radicular son principalmente para incrementar el área de exploración de la raíz y de esta forma obtener él o los nutrientes que requiere la planta para su óptimo crecimiento (López-Bucio *et al*., 2003; Vance, 2008). En *Arabidopsis* se ha descrito la vía de señalización que está involucrada en la modificación de la arquitectura radicular en condiciones deficientes de P (Sánchez-Calderón *et al*., 2005). Entre los 600 genes que respondieron diferencialmente a la deficiencia de P en nódulos y raíces de frijol, se detectaron pocos que tienen una probable participación en este proceso en frijol. Lo anterior puede deberse a que en esta leguminosa no se conoce por completo su genoma, lo cual limita las probabilidades de encontrar bioinformática y experimentalmente genes asociados a la modificación de la arquitectura radicular. Aunado a lo anterior, los macroarreglos que se usaron en el presente trabajo fueron impresos con EST provenientes de raíces deficientes de P y de nódulos de frijol, lo cual limitó aun más la identificación de genes asociados a este proceso. Probablemente, el análisis de las respuestas de raíces limitadas en P a tiempos más cortos que los analizados en este trabajo podrá revelar más genes asociados a la modificación de la arquitectura radicular.

La mayoría de las respuestas bioquímicas al estrés nutricional están asociadas a la modificación del flujo de carbono/nitrógeno y a la homeostasis nutricional (Udhe-Stone *et al*., 2003; Vance, 2008). En la deficiencia nutricional estas modificaciones metabólicas ayudan a la planta a removilizar y transportar los nutrientes, tanto de fuentes internas (biomoléculas) como de fuentes externas (rizosfera) (Rhagothama, 1999, 2000; Vance, 2008). En cambio, en la toxicidad nutricional estas respuestas están

orientadas a empaquetar y/o excretar los excesos de él o de los nutrientes (Hall, 2003; Pittman, 2005). En este trabajo, con el análisis transcripcional de las respuestas de frijol a la deficiencia de P y a la toxicidad por Mn^{+2} identificamos diversos genes que están asociados al flujo de carbono/nitrógeno, en la síntesis de ácidos orgánicos y en la homeostasis de estos dos nutrientes. Por ejemplo, en la toxicidad de Mn^{+2}, encontramos que la expresión de genes asociados a la asimilación de nitrógeno (*PvGS*) y al flujo de carbono (*PvG3PDH*), se redujo, lo cual concuerda con los parámetros de FSN analizados en este trabajo. Además, encontramos que *SNARE*, un gen involucrado en el sistema de transporte del aparato de Golgi (Collins *et al*., 2003), se indujo en nódulos de plantas de frijol tratados con excesos de Mn^{+2}. Al respecto, se ha reportado que durante la toxicidad por Mn^{+2} los excesos de este ión son exudados o empaquetados en distintos organelos, como el aparato de Golgi (Pittman, 2005), por lo que este gen podría participar en la desintoxicación y en la homeostasis de Mn^{+2} en nódulos. Con respecto a la deficiencia de P, mediante sRT-PCR y Northern Blot, identificamos que la expresión de distintos genes que participan en la homeostasis de P (*Pv4*, miR399, *PHO2*, *Pth1*) se modificó durante la deficiencia de este nutriente solo en raíces y no en nódulos. Asimismo, mediante el análisis integral de datos de transcriptoma y metaboloma, pudimos identificar los metabolitos y rutas metabólicas que se modifican en raíces y nódulos deficientes de fósforo. Interesantemente, el set de genes y el patrón de acumulación de metabolitos registrado en raíces deficientes de P fueron diferentes a lo registrado en nódulos. Por ejemplo, en raíces de frijol deficientes de P se registró un incremento de distintos azucares y una disminución de ácidos orgánicos, lo cual también ha sido registrado en *Arabidopsis* (Morcuende *et al*., 2007). En contraste, en nódulos de frijol deficientes de P se registró un efecto totalmente contrario, en el cual se acumularon distintos ácidos orgánicos. Este comportamiento también se reportó en nódulos de *Lupinus albus* deficientes de P y se asoció con la disminución en la fijación de nitrógeno (Le Roux *et al*., 2008), que también se observó en frijol. En conjunto, estos datos sugieren que las raíces y nódulos de plantas de frijol responden de forma distinta a cada estrés nutricional.

Durante el estrés nutricional, ya sea por deficiencia o toxicidad, se induce la producción de especies reactivas de oxígeno (ROS) que conllevan al estrés oxidativo (Hall, 2002). Este efecto en la deficiencia nutricional, por ejemplo, en la deficiencia de P, puede deberse a los ajustes en el transporte de electrones fotosintético/respiratorio que se llevan a cabo para evitar el uso "excesivo" de P (Vance, 2008). En la toxicidad nutricional, la producción de ROS es debido a que se afectan negativamente a distintos acarreadores de energía tanto a nivel respiratorio como fotosintético, lo cual genera un desequilibrio redox que es favorable para la formación de ROS (Hall, 2002). En el presente trabajo identificamos genes de distintas peroxidasas que respondieron a la deficiencia de P y toxicidad de Mn^{+2} en raíces y nódulos. Observamos en nódulos de plantas de frijol sometidas a toxicidad extrema de Mn^{+2}, la actividad de la CAT y SOD se incrementó en más de tres veces con respecto al control, lo cual concuerda con lo reportado por González y Lynch (1997) en hojas de frijol. Estos datos indican que los genes de peroxidasas detectadas en la deficiencia de P y toxicidad por Mn^{+2}, así como la CAT y SOD en nódulos de plantas estresadas con Mn^{+2}, pueden tener un papel relevante en la defensa contra el estrés oxidativo que induce la deficiencia de P y toxicidad por Mn^{+2}.

En distintos análisis de perfiles de transcripción de plantas deficientes de P, se han observado que los FT que responden a este estrés están asociados al mantenimiento de la homeostasis y del control de la modificación de la arquitectura radicular (Misson *et al*., 2005; Hernández *et al*., 2007; Morcuende *et al*., 2007). Un comportamiento

similar fue registrado en el presente trabajo; sin embargo, en nódulos de frijol deficientes de P observamos que la mayoría de los FT que respondieron a este estrés pueden estar asociados con la modificación de la arquitectura radicular (*NAC, bHLH, AUX/IAA, C2C2(Zn)*) y en el desarrollo y mantenimiento de la función de los nódulos (*AP2/EREBP*) (Chen *et al*., 2007; Devaiah, 2007; Asamizu *et al*., 2008). Es interesante mencionar que la familia AP2/EREBP es una de las más sobre-representadas en los transcriptomas de diferentes leguminosas (Benedito *et al*., 2008), por lo que miembros de esta familia de FT pueden jugar un papel importante en la adaptación de los nódulos a distintos estreses nutricionales.

En la deficiencia y toxicidad nutricional se ha reportado el entrecruzamiento de vías de señalización que están vinculadas a otros estreses bióticos y/o abióticos (Fecht-Christoffers *et al*., 2003; Franco-Zorrilla *et al*., 2004; Führs *et al*., 2008). Por ejemplo, en un análisis del proteoma de hojas de plantas de *Vigna unguiculata* tratadas con exceso de Mn^{+2} se encontró que varias de las proteínas que respondieron a este estrés estaban asociadas a patogénesis (Fecht-Christoffers *et al*., 2003; Führs *et al*., 2008). Nuestro análisis de transcriptoma de raíces y nódulos deficientes de P y de nódulos tratados con dos concentraciones de Mn^{+2}, reveló la presencia de genes asociados a patogénesis y otros estreses abióticos. En general, los datos encontrados en este trabajo demuestran que en las respuestas a la deficiencia de P y toxicidad por Mn^{+2} el entrecruzamiento de distintas vías de señalización puede ser vital en la adaptación y sobrevivencia de las plantas de frijol a estos dos estreses nutricionales. Este entrecruzamiento de vías de señalización puede deberse a que se generan distintas moléculas señal o simplemente la que se genera puede ser común a distintos estreses. La demostración y caracterización de la ó las moléculas señal podría ser de utilidad en la comprensión de las respuestas de frijol a distintos estreses nutricionales.

Recientemente en *Arabidopsis* se ha reportado la participación de miRNAs en distintos procesos de desarrollo de la planta (Boutet *et al*., 2003; Lobbes, *et al.*, 2006; Yang *et al*., 2006; Yu *et al*., 2008; Gregory *et al*., 2008), así como en la deficiencia de N_2 (miR167), Cu^{+2} (miR397/miR398/miR408/miR857), azúfre (S) (miR395) y P (miR399/miR144/miR827) (Bari *et al*., 2006; Abdel-Ghany & Pilon, 2008; Gifford *et al*., 2008; Kawashima *et al*., 2009; Pant *et al*., 2008, 2009;). Además, en leguminosas se ha reportado la participación de miRNAs en el desarrollo del nódulo, en la adaptación de frijol al estrés por sequía y por salinidad, así como en la homeostasis de P (Combier *et al*., 2006; Boualem *et al*., 2008; Arenas-Huertero *et al*., 2009; Valdés-López *et al*., 2008). En este trabajo reportamos 32 miRNAs, incluyendo miRNAs conservados y específicos de soya y frijol, que tienen una probable participación en el desarrollo de hojas, raíces y nódulos de frijol. De igual forma, reportamos 35 miRNAs que responden a la deficiencia de N, Fe^{+2}, P, a pH ácido (pH5.5) y a la toxicidad de Mn^{+2}. Algunos de los miRNAs que respondieron a estos estreses nutricionales ya habían sido vinculados con otros estreses abióticos, lo que sugiere que estos miRNAs pueden estar involucrados en el entrecruzamiento de vías de señalización. Sin embargo, la mayoría de los miRNAs que respondieron a la deficiencia nutricional y a la toxicidad por Mn^{+2} solo habían sido vinculados a distintos procesos de desarrollo (Boutet *et al*., 2003; Lobbes, *et al.*, 2006; Yang *et al*., 2006; Yu *et al*., 2008; Gregory *et al*., 2008). Muy pocos de estos miRNAs pudieron ser asociados con los cambios morfológicos que experimenta frijol en este tipo de estreses. Por ejemplo, en nódulos de plantas de frijol sometidos a toxicidad moderada y extrema de Mn^{+2} observamos que miR156/miR157 incrementó su abundancia, mientras que el nivel de *PvSPL6* (mRNA blanco) mostró una disminución, sugiriendo que miR156/miR157 son funcionalmente activos en este estrés. Sin embargo, la información disponible sobre este miRNA indica que participa en el

desarrollo de flor y no en procesos de nodulación o en condiciones de estrés (Gandikota *et al*., 2007). Dado que la mayoría de los miRNAs que encontramos en este trabajo han sido vinculados en procesos de desarrollo y no a ningún estrés abiótico, en este caso nutricional, se proponen dos escenarios: 1) estos miRNAs y sus respectivos blancos pueden tener otra función adicional a la ya descrita, o bien 2) que estos miRNAs pueden tener más blancos de los descritos hasta el momento, lo cual puede ser viable puesto que la mayoría de los algoritmos pueden subestimar el número real de mRNAs blancos que tiene un miRNAs dado (Franco-Zorrilla *et al*., 2009; Voinnet, 2009).

En general, los datos obtenidos en este trabajo pueden representar la base para poder entender las respuestas de frijol al estrés nutricional, particularmente en deficiencia de P y toxicidad por Mn^{+2}. Asimismo, estos resultados pueden ser la justificación para analizar individualmente alguno de los genes que respondieron a la deficiencia de P y toxicidad por Mn^{+2}. Por ejemplo, los datos derivados de nuestro análisis bioinformático y del transcriptoma de frijol sirvieron para poder trazar la primera ruta de señalización transcripcional y post-transcripcional de raíces de frijol deficientes de P (Valdés-López *et al*., 2008). Aunado a esto, la descripción de esta vía de señalización también demuestra que el análisis de genética reversa es viable en plantas compuestas de frijol.

V. CONCLUSIONES Y PERSPECTIVAS

En esta tesis doctoral se analizaron los transcriptomas de raíces y nódulos deficientes de P, así como de nódulos tratados con excesos de Mn^{+2}. Además se identificaron distintos reguladores transcripcionales y post-transcripcionales, algunos de ellos no habían sido reportados en otras especies vegetales tratadas con los mismos estreses aplicados en este estudio. Con estos resultados podemos concluir:

- Las raíces y nódulos poseen diferentes programas transcripcionales y respuestas metabólicas para hacer frente a un mismo estrés nutricional.
- Las respuestas transcripcionales y metabólicas de raíces deficientes de P están orientadas a mantener la homeostasis de P, mientras que las de nódulos en su mayoría están asociadas con el flujo y la asimilación de C/N.
- La vía de señalización regulada por PHR1 opera en raíces de frijol deficientes de P. A diferencia de *Arabidopsis*, esta vía puede ser la principal en regular los genes asociados a la homeostasis de P.
- La toxicidad por Mn^{+2} afecta negativamente la FSN a través de la reducción del número de células infectadas, de la actividad de la nitrogenasa y del contenido de Fe^{+2} en nódulos.
- CAT y SOD incrementaron su actividad en nódulos de plantas de frijol sometidas a toxicidad extrema por Mn^{+2}, sugiriendo que la toxicidad por Mn^{+2} induce estrés oxidativo en nódulos.
- Algunas de las respuestas transcripcionales a la toxicidad por Mn^{+2} están asociadas a la regulación del metabolismo de C/N, en la defensa al estrés oxidativo y en la homeostasis de Mn^{+2}.
- Distintos miRNAs, la mayoría de ellos previamente asociados a procesos de desarrollo, responden diferencialmente a la deficiencia de N, P, Fe^{+2}, pH5.5 y toxicidad por Mn^{+2}. Estos datos representan el primer reporte de los miRNAs que responden a la deficiencia de Fe^{+2} y toxicidad por Mn^{+2}.

Los resultados obtenidos en esta tesis doctoral abren la posibilidad de:

- Identificar las vías de señalización que controlen las respuestas a la deficiencia de P y toxicidad por Mn^{+2}. Por ejemplo, resultaría interesante determinar el papel que juega el regulador transcripcional TIFY y el FT AP2/EREBP en la adaptación de las raíces y nódulos a la deficiencia de P.
- Determinar el papel fisiológico de *PvSNARE* y *PvVSP* durante la toxicidad de Mn^{+2}, lo cual implicaría determinar su perfil de expresión en distintos órganos de frijol y en distintas concentraciones de Mn^{+2}; determinar si su respuesta es específica a la toxicidad por Mn^{+2}; determinar su localización subcelular. Una vez analizados estos parámetros, se tendría que analizar algunas propiedades bioquímicas y analizar plantas compuestas de frijol que tengan, por separado, estos genes silenciados o sobre-expresados.
- Determinar si la disminución de la actividad de la nitrogenasa registrada en nódulos de plantas tratadas con exceso de Mn^{+2} se debe a la disminución del complejo Fe-Mo, o en su defecto determinar las causas precisas de este comportamiento.
- Determinar el papel fisiológico de los miRNAs que respondieron a la deficiencia nutricional (-P, -N y –Fe) y a la toxicidad por Mn^{+2}. Los candidatos que resultaría interesante analizar son aquellos que previamente habían sido vinculados a procesos de desarrollo, porque implicaría determinar si estos miRNAs tienen otros blancos, adicionales a los ya descritos, o si los blancos descritos tienen otras funciones. Además, el análisis de la función de miRNAs en estrés nutricional implicaría determinar la localización subcelular de los miRNAs y los mRNAs blancos, hacer análisis de ganancia y pérdida de función de cada uno de ellos.

VI. REFERENCIAS BIBLIOGRÁFICAS

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Identification of candidate phosphorus stress induced genes in *Phaseolus vulgaris* through clustering analysis across several plant species

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Abstract. Common bean (*Phaseolus vulgaris* L.) is the world's most important grain legume for direct human consumption. However, the soils in which common bean predominate are frequently limited by the availability of phosphorus (P). Improving bean yield and quality requires an understanding of the genes controlling P acquisition and use, ultimately utilising these genes for crop improvement. Here we report an *in silico* approach for the identification of genes involved in adaptation of *P. vulgaris* and other legumes to P-deficiency. Some 22 groups of genes from four legume species and *Arabidopsis thaliana*, encoding diverse functions, were identified as statistically over-represented in EST contigs from P-stressed tissues. By combining bioinformatics analysis with available micro / macroarray technologies and clustering results across five species, we identified 52 *P. vulgaris* candidate genes belonging to 19 categories as induced by P-stress response. Transport-related, stress (defence and regulation) signal transduction genes are abundantly represented. Manipulating these genes through traditional breeding methodologies and / or biotechnology approaches may allow us to improve crop P-nutrition.

Keywords: ESTs sequences, genomics, legumes, phosphate deficiency, stress.

Introduction

Building the foundations for common bean functional genomics

Common bean is the world's most important grain legume for direct human consumption. In Mexico, and other countries of Central and South America, beans are staple crops serving as the primary source of protein N in the diet (Broughton *et al*. 2003; Graham *et al*. 2003). In Latin America and Africa, the yield of bean production is low, in part because of disease and insect pressures but also because of edaphic constraints that include soil N and P deficiencies, soil acidity, and aluminum, manganese, and iron toxicities (Graham 1981; Graham *et al*. 2003). It has been suggested that 89% of soils in Latin America are deficient in N and 82% are deficient in P, with more than 500 million Ha of soil in this region with a pH of 4.5 or less (Sánchez and Cochrane 1980). Overcoming edaphic stresses and improving crop yield are high-priority goals. Identification of the plant genes involved in these processes will not only increase our knowledge of processes integral to crop productivity, but also will identify new targets for crop improvement.

Despite the importance of common bean as a crop legume, very little expressed sequence tag (EST) information is publicly available. In efforts to develop molecular tools and to provide an initial platform for comparative

Abbreviations used: EST, expressed sequence tag; P, phosphorus; TIGR, The Institute for Genomic Research.

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functional genomics, we initiated a collaborative project on common bean ESTs sequencing. We previously reported the sequencing and assembly of 21 026 ESTs derived from two *P. vulgaris* genotypes — Mesoamerican Negro Jamapa 81 and Andean G19833. ESTs were derived from root nodules, roots from P-deficient plants, developing and mature pods, and leaves (Ramírez *et al*. 2005). Recently, Melotto *et al*. (2005) reported the sequencing of 5255 ESTs from *P. vulgaris* genotype SEL1308. These sequences were derived from two cDNA libraries developed from leaves and seedlings, inoculated or non-inoculated with the fungal pathogen *Colletotrichum lindemuthianum*. Here we report the *P. vulgaris* gene index elaborated after the combined contig (the consensus sequence for an assembly of ESTs corresponding to a given gene) analysis of all the common bean ESTs publicly available.

Identification of genes important in P acquisition

Recent advances in macro- and microarray technology have led to the identification of several genes involved in plant responses to P deficiency. High-density macroarray analysis was performed to evaluate gene expression in response to P-deficiency in white lupin (*Lupinus albus*, Uhde-Stone *et al*. 2003). White lupin adapts to P deficiency by the development of short, densely clustered lateral roots called proteoid roots. Nylon filter arrays with some 2000 ESTs from proteoid roots were performed to identify genes differentially expressed in P-deficient proteoid roots as compared to normal roots. Some 35–40 genes that are more highly expressed in −P cluster roots than in + P roots were identified, including genes involved in carbon and secondary metabolism, P scavenging and remobilisation, plant hormone metabolism, and signal transduction (Uhde-Stone *et al*. 2003).

More recently, a comprehensive survey of gene expression in response to P deprivation in *Arabidopsis thaliana* has been reported (Misson *et al*. 2005). For this study the whole-genome Affymetrix gene chip (ATH1) was used to quantify the spatio-temporal variations in transcript abundance of some 22 000 genes. Analysis of short-, medium-, and long-term P deprivation revealed a total of 866 differently expressed genes; 612 of these were induced. Genes involved in several biochemical pathways that are closely associated with plant responses to P deficiency were coordinately activated and repressed. The functional classification of the differentially expressed genes also included those involved in ion transport, signal transduction, transcriptional regulation, and growth and development processes (Misson *et al*. 2005).

Identification of genes important in phosphate stress across species

Datasets that identify ESTs responsive to P-deficiency have been developed in several species. In *Medicago truncatula*, three EST libraries (totaling 13 245 ESTs) are available from P-starved roots and leaves [The Institute for Genomic Research (TIGR), http://www.tigr.org; verified 6 July 2006]. In soybean (*Glycine max* and *G. sojae*), 5 429 ESTs are available from P-starved roots (TIGR). In *L. albus*, 3260 ESTs are available from different stages of P-starved proteoid roots (Uhde-Stone *et al*. 2003 and C Vance unpubl. data). In addition, we have generated 3165 ESTs from P-starved roots of *P. vulgaris* (Ramírez *et al*. 2005). In this report, we use statistical and cluster analyses of EST data to identify candidate genes potentially involved in P-starvation from *P. vulgaris* and other legumes. Through *in silico* analysis of ESTs from *P. vulgaris*, *M. truncatula*, soybean, *L. albus*, and *A. thaliana*, we have identified P-stress genes that are statistically over-represented. Genes identified from *P. vulgaris* will build a foundation for future research.

Material and methods

Processing and contig assembly of P. vulgaris *and* L. albus *ESTs*

To analyse the most complete *Phaseolus vulgaris* L. dataset possible, the ESTs sequenced by Ramírez *et al*. (2005) and Melotto *et al*. (2005) were considered together. EST sequences generated by Melotto *et al*. (2005) were downloaded from DbEST (http://www.ncbi.nlm.nih.gov/dbEST/; verified 6 July 2006). The sequences of both groups were assembled with a processing pipeline developed by the Center for Computational Genomics and Bioinformatics (CCGB) at the University of Minnesota, as described by Ramírez *et al*. (2005). The results of this analysis are shown in Tables 1 and 2.

Uhde-Stone *et al*. (2003) sequenced 2102 ESTs from 7- to 10-d-old and 12- to 14-d-old P-starved proteoid roots of *Lupinus albus* L. An additional 1140 ESTs have recently been generated from emerging proteoid roots (C Vance unpubl. data). Using the processing pipeline mentioned above, we assembled the *L. albus* ESTs into 409 contigs and and 1379 singletons (data not shown).

Identification of contigs statistically over-represented with ESTs from P-starved tissues

To identify genes important under conditions of phosphate stress, TIGR's soybean (GmGI version 12) and *Medicago truncatula* Gaertn. (MtGI version 8) gene indices were searched for EST libraries derived from phosphate-starved tissues (http://www.tigr.org/tdb/tgi/plant.shtml; verified 6 July 2006). Three libraries were identified from *M. truncatula*. MHRP- and rootphos(-), containing 2649 and 1953 ESTs respectively, were derived from P-starved roots; NF–PL, containing 8643 ESTs, was derived from P-starved leaves. In soybean, only a single phosphate-starved root library was identified (5429 ESTs). In addition to TIGR's gene indices, we also took advantage of the 3165 P-stressed root ESTs available from *P. vulgaris* (Ramírez *et al*. 2005).

Table 1. Contigging statistics of *Phaseolus vulgaris* ESTs

Tissues	ESTs in contigs >1	EST Singletons
MesoAmerican nodules	2537	1208
MesoAmerican pods	2043	904
MesoAmerican roots	1882	1283
MesoAmerican leaves	2072	605
Andean leaves (5′ and 3′)	2075	1168
Shoot (Sel 1308)	1667	802
Shoot (Sel 1308) inoculated with *Colletotrichum*	1515	813
Total ESTs	13 791	6787

Table 2. Characteristics of tissue-specific contigs from *Phaseolus vulgaris* ESTs

Tissue-specific contigs	Number of contigs >1	Average ESTs per contig	Average length	Largest contig (no. ESTs)
MesoAmerican nodule-specific	207	2.5	785.6	10
MesoAmerican pod-specific	87	3.9	748.3	64
MesoAmerican root-specific	190	2.5	736.6	11
MesoAmerican leaves-specific	29	2.5	751.4	8
Andean leaves-specific	121	2.9	814.9	26
Shoot (Sel 1308)	36	2.1	617.5	3
Shoot (Sel 1308) inoculated with *Colletotrichum*	40	2.2	575.5	7
Mixed tissue contigs	2173	5.5	897.3	269
All contigs	2883	4.8	861.3	269

Custom perl scripts were used to examine each contig / phosphate-starved library combination. For most species, each EST contig was examined only once, since only one P-starved EST library was available. For *M. truncatula*, each contig was examined three times, once for each of the three P-starved libraries. For each contig / library combination, a perl script was used to count four observed values: the number of P-starved ESTs from a particular library in and out of the contig and the number of 'other' ESTs in and out of the contig. 'Other' ESTs included all other EST libraries except those from other P-starved libraries, those whose tissue origin could not be determined, or those representing resequenced libraries.

Based on the four observed counts, a second perl script calculated the expected values based on EST frequency. If the observed and expected counts within a contig were greater than four, a chi-square association test (Dunn and Clark 2001) determined the statistical significance of the results. If any of the counts was less than four, statistical significance was calculated by the Fisher exact probability test (Siegel 1956). Each of these tests determined whether a contig has a true over-representation of P-starved ESTs or if the number observed could occur by chance. The Fisher exact test was used with counts less than four because it won't exaggerate probability estimates. If the probability obtained for a particular contig / library combination was less than or equal to 0.05, a contig / library combination was considered statistically over-represented with ESTs from the corresponding P-starved library. Using this approach a single contig could be statistically over-represented with P-starved ESTs from more than one library. An example of the analysis is shown in Fig. 1.

In the case of *L. albus*, all available ESTs came from P-starved proteoid root tissues. Since ESTs from other tissues were not available, statistical analysis of *L. albus* contigs could not be performed. However, these genes were included in our clustering analyses because they were derived from P-starved roots and many of the genes have been confirmed to show enhanced expression under P-starvation with macroarray analysis (Uhde-Stone *et al*. 2003; S Miller, C Vance unpubl. data). Results from *L. albus* macroarray and *Arabidopsis* microarray experiments (Misson *et al.* 2005) provide strong support for bioinformatics approaches.

Identification of A. thaliana *genes induced in leaves and roots of P-starved tissues*

Misson *et al*. (2005) used the *Arabidopsis thaliana* (L.) Heyhn. whole-genome Affymetrix gene chip (ATH1) to identify *A. thaliana* genes induced or repressed during P-starvation. In P-starved leaves, 404 genes were significantly (probability <0.05) up-regulated with at least 2-fold induction. In P-starved roots, 231 genes were significantly up-regulated with a minimum 2-fold induction of gene expression. In total, 494 unique genes were up-regulated during P-starvation in leaves and / or roots. The gene identifiers were used to download the corresponding sequences from The *Arabidopsis* Information Resource (TAIR, http://www.arabidopsis.org; verified 6 July 2006).

Single linkage clustering of homologous sequences across species

The sequences of statistically over-represented P-starved contigs from *P. vulgaris*, soybean, and *M. truncatula*, the *A. thaliana* P-starvation statistically up-regulated genes identified by Misson *et al*. (2005), and the sequences of the 409 contigs assembled from P-starved proteoid roots of *L. albus* ESTs were combined to give a dataset totalling 2097 sequences (Table 3). Genes important in P-stress across species were identified by a two-step approach. TBLASTX (Altschul *et al*. 1997) of the dataset against itself was used to identify homologous sequences (E-value cutoff of 10^{-4}). Single linkage clustering, described by Graham *et al*. (2004), assigned homologous sequences into groups. Groups that only contained sequences from *L. albus* were deleted as no statistical analyses had been performed on these sequences. Once group identifiers were assigned, all sequences in each group were compared to the Uniprot protein database (Apweiler *et al*. 2004) using BLASTX (Altschul *et al*. 1997) and an E-value cutoff of 10^{-4}. These results were used in functional annotation of the groups.

Results

Phaseolus vulgaris *gene index*

We previously reported sequencing five EST libraries from *P. vulgaris*. Since then, an additional 5255 ESTs have been deposited in DbEST (http://www.ncbi.nlm.nih.gov/dbEST/) by Melotto *et al*. (2005). The combination of these two datasets provides a single *P. vulgaris* gene index containing 20 578 ESTs. Of these, 6787 were classified as singletons and the remaining 13 791 assembled into 2883 contigs ranging in EST redundancy from 2 to 269 (Table 1) resulting in a 9670 unigene set. Library specific contigs ranged from 29 to 207 (Table 2). Data from this new build can be downloaded from our website (http://www.ccg.unam.mx/phaseolusest/; verified 6 July 2006).

Identification of contigs statistically over-represented with ESTs from phosphate-starved tissues

Using algorithms and statistical analysis we evaluated 31 928 contigs from soybean, 18 612 contigs from *M. truncatula*, and 2883 contigs from *P. vulgaris* for statistical over-representation of ESTs from P-starved libraries (Table 3).

The number of contigs that were over-represented under P-stress conditions ($P<0.05$) in the three species ranged from 247 to 543. In the case of *L. albus*, the 409 contigs used for cluster analysis came from P-stress-induced proteoid roots. These *L. albus* contigs have been used in macroarray analysis to identify genes induced during P-starvation. The 494 contigs up-regulated due to P-stress in *Arabidopsis* and used in our cluster analysis were identified by Misson *et al*. (2005).

A ***Phaseolus vulgaris* contig 3247**

Observed values	ESTs in contig	ESTs out of contig	Total ESTs
P-starved root ESTs	38.00	3150.00	3188.00
Other ESTs	4.00	17 595.00	17 599.00
Total ESTs	42.00	20 745.00	20 787.00
Expected values	**ESTs in contig**	**ESTs out of contig**	**Total ESTs**
P-starved root ESTs	6.44	3181.56	3188.00
Other ESTs	35.56	17 563.44	17 599.00
Total ESTs	42.00	20 745.00	20 787.00
χ^2 analysis	**χ^2 in contig**	**χ^2 out of contig**	**Total χ^2**
P-starved root ESTs	154.63	0.31	154.94
Other ESTs	28.01	0.06	28.07
Total χ^2	182.63	0.37	183.00
Degrees of freedom	1		
Probability	1.07E–41		

B **Representatives of Group 179**

Sequence name[A]	Statistically significant tissue	*P*-value
At4g12470[B]	P-starved leaves	5.00E–02
MtTC100494	P-starved leaves	9.98E–03
MtTC100581	P-starved roots	2.63E–02
MtTC106613	P-starved roots	2.59E–03
PvContig1804	P-starved roots	2.35E–02
PvContig2421	P-starved roots	5.52E–04
PvContig2964	P-starved roots	1.98E–06
PvContig3247	P-starved roots	1.07E–41

[A]The two letters in front of the name are species identifiers: At, *A. thaliana*; Mt, *M. truncatula*; Pv, *P. vulgaris*.

[B]Identified in microarray analyses by Mission *et al*. (2005). Significance $P<0.05$.

Clustering of P-starvation-induced genes across species

One of the negative aspects of the statistical approach described above is that the number of EST libraries available for a given species may impact the results. For example, in each gene, the analyses would examine the number of ESTs derived from P-starved tissues compared with the number of ESTs derived from other tissues. For example, in *M. truncatula* and soybean, ESTs from P-starved roots were compared to all other ESTs including untreated roots. Genes identified as statistically over-represented in P-starved roots are likely a response to P-starvation. In contrast, in *P. vulgaris* no ESTs were available from untreated roots. Therefore, the statistically over-represented sequences we identified may be the result of overexpression in roots in general and may not reflect a response to P starvation (see Fig. 1).

To aid in identifying P-starvation-induced genes from *P. vulgaris* and to identify genes conserved across species, we clustered ESTs statistically over-represented from *P. vulgaris* with those from *M. truncatula* and soybean. In addition, we included statistically significant P starvation-induced genes identified from *A. thaliana* microarrays (Misson *et al*. 2005) and ESTs developed from P-starved *L. albus* roots for which additional macroarray data are available (Uhde-Stone *et al*. 2003). We hypothesised that if a candidate gene identified from *P. vulgaris* was also induced in response to P-starvation in other species, it may be a high priority candidate for future research. Single-linkage clustering was used to assemble the 2097 P-starved sequences from *P. vulgaris*, *M. truncatula*, soybean, *A. thaliana*, and *L. albus* into 287 sequence-homology based groups. Groups ranged in size from 2 to 98 sequences and had representatives from 1–5 species. Groups of genes that were

Fig. 1. Identification of genes expressed in response to P-starvation across species. (*A*) In this example from *P. vulgaris* contig 3247, a chi-square association test (Dunn and Clark 2001) was used to determine whether P-starved ESTs were statistically over-represented within the contig. In the first step, observed values are reported for ESTs assembled in the contig and ESTs outside of the contig. In the second step, the frequency of P-starved ESTs overall was used to calculate expected values for ESTs assembled in and out of the contig. The chi-square test then measured if the observed values were significantly different from the expected values. (*B*) To further limit the number of candidate genes identified, single linkage clustering was used to identify sequences that were homologous to *P. vulgaris* contig 3247 and were also over-represented during P-starvation. While the sequences from *M. truncatula* and *P. vulgaris* were identified by the bioinformatic methods described above, the *A. thaliana* sequence was identified by Misson *et al*. (2005) as significantly up-regulated in response to P starvation from microarray experiments.

Table 3. The number of genes (contigs) from five plant species examined to derive those that are statistically over-represented in libraries from P-deficient tissues

Species	Genes / contigs examined[A]	Statistically over-represented genes / contigs[B]
P. vulgaris	2883	247
Soybean	31 928	543
M. truncatula	18 612	404
A. thaliana	494	494
L. albus	409	409
Sum	54 326	2097

[A]In order to identify genes involved in P-starvation, we used sequences from a variety of sources. For *Phaseolus vulgaris* and *Lupinus albus*, contigs from the most recent assembly were used for analysis (see Materials and methods). For soybean and *Medicago truncatula*, TIGRs GmGI (version 12) and MtGI (version 8) gene indices were used. For *Arabidopsis thaliana*, sequences identified by Misson *et al*. (2005) were used for analysis.
[B]For *Phaseolus vulgaris*, soybean, and *Medicago truncatula*, statistical analyses were performed by chi-square association (Dunn and Clark 2001) or Fisher exact tests (Siegel 1956). No statistical analyses were performed on *Lupinus albus* sequences. For *Arabidopsis thaliana*, Misson *et al.* (2005) identified genes statistically up-regulated during P-starvation (probability <0.05) with a 2-fold increase in expression in P-starved roots and / or leaves.

over-represented in four or five species are listed in Table 4. Genes found to be over-represented in four or five species from P-stressed libraries were considered as important candidates involved in adaptation to P-deficiency.

Can bioinformatic analyses be used to identify candidate genes involved in P stress from P. vulgaris*?*

The 22 groups of genes listed in Table 4 are over-represented in P-stressed conditions and are likely candidates to be involved in plant responses to P stress. Of the 22 groups, 20 include *A. thaliana* sequences identified by Misson *et al*. (2005) as significantly induced in response to P stress. In addition, eight of the groups are supported by macroarray data of *L. albus* P-stress-induced genes identified by Uhde-Stone *et al*. (2003). *Phaseolus vulgaris* sequences are found in 19 of the 22 groups. By combining our bioinformatic analyses with available micro / macro array technologies and clustering results across species, we identified 52 *P. vulgaris* sequences (represented in the 19 groups) (Table 5). Two of these *P. vulgaris* genes are induced in P-deficient roots from bean plants, as shown by RNA-blot analysis (Ramírez *et al*. 2005). The genes noted in Table 5 will be priority targets for future research.

Table 4. Single linkage clustering identifies homologous sequences across species that are important in response to phosphate starvation
Group numbers are assigned at random. The data in this table correspond to groups containing sequences from four or five species. Two-letter designators are used to describe the species found in each group. At refers to *Arabidopsis thaliana*, Mt refers to *Medicago truncatula*, Gm refers to soybean, La refers to *Lupinus albus*, and Pv refers to *Phaseolus vulgaris*. All Pv, Mt, and Gm sequences were identified as statistically over-represented in P-starved tissues. All *Arabidopsis thaliana* sequences were significantly up-regulated in response to P stress (Misson *et al.* 2005; microarray data). All *Lupinus albus* sequences designated by an asterisk (*) were identified as significantly up-regulated in response to P stress in macroarray experiments (Uhde-Stone *et al.* 2003). *Phaseolus vulgaris* sequences designated by two asterisks (**) were identified as induced in roots from P-starved plants, by RNA-blot analysis (Ramírez *et al*. 2005). Annotations were assigned by comparing all sequences in the group to the Uniprot database using TBLASTX and a cut-off of $E < 10^{-4}$

Group number	Species represented	Sequences in group	Group annotation
0	At Mt Gm La* Pv**	17	Aquaporin
2	At Mt Gm La Pv	6	Pectin methylesterase
11	At Mt Gm La* Pv	98	Protein kinase
17	At Mt Gm La Pv	30	Peroxidase
18	At Mt Gm La Pv	22	ABC transporter family
42	At Mt Gm La Pv	12	WRKY transcription factor
56	At Mt Gm La* Pv	23	Cytochrome P450
77	At Mt Gm La* Pv	14	Oxygenase
14	At Gm La Pv**	6	Protein phosphatase 2C
15	Mt Gm La Pv	9	RAB protein
21	At Mt Gm La*	11	Phosphate transporter
31	At Gm La Pv	10	MYB transcription factor
57	At Gm La Pv	5	Dihydroflavonol or cinnamoyl reductase
62	At Gm La Pv	4	Proline-rich extensin
63	Mt Gm La* Pv	7	Glyceraldehyde 3 phosphate dehydrogenase
64	At Mt Gm La*	21	Purple acid phosphatase
74	At Mt Gm Pv	7	4-Coumarate-CoA ligase-like
79	At Mt Gm Pv	6	Zinc finger protein
84	At Gm La Pv	10	Glycosyl hydrolase
179	At Mt La* Pv	14	Class 10 PR protein
257	At Mt La Pv	4	Cyclic nucleotide-binding transporter
286	At La Mt Pv	31	Chlorophyll *a* / *b* binding protein

Table 5. Candidate *Phaseolus vulgaris* genes identified as likely to be relevant for response to P starvation

Group number	Contig name	Contig length	Probability of over-representation in P-starved roots	Top Uniref100 TLASTX B	E-value
0	PvContig2458	997	5.52E–04	Q41975 Probable aquaporin TIP2.2 (*A. thaliana*)	1.00E–89
0	PvContig3195	1297	2.90E–06	Q506K1 Putative aquaporin (*P. vulgaris*)	1.00E–161
2	PvContig1817	797	2.35E–02	O04887 Pectinesterase-2 precursor (*C. sinensis*)	1.00E–111
11	PvContig545	946	2.35E–02	Q9C753 Serine / threonine kinase, putative (*A. thaliana*)	1.00E–148
11	PvContig918	447	2.35E–02	Q9SII6 Hypothetical protein At2 g17220 (*A. thaliana*)	6.00E–16
11	PvContig1048	750	2.35E–02	Q9MBG5 Similarity to calmodulin (*A. thaliana*)	5.00E–24
11	PvContig1180	797	2.35E–02	Q5XWQ1 Serine / threonine protein kinase-like (*S. tuberosum*)	2.00E–96
11	PvContig1785	658	2.35E–02	Q9M1Q2 Serine / threonine protein kinase-like protein (*A. thaliana*)	1.00E–61
11	PvContig1788	952	2.35E–02	Q5JCL0 Mitogen-activated protein kinase kinase MAPKK2 (*G. max*)	1.00E–135
11	PvContig1805	617	2.35E–02	O49840 Protein kinase (*A. thaliana*)	1.00E–45
11	PvContig2010	721	3.60E–03	Q9SCZ4 Receptor-protein kinase-like protein (*A. thaliana*)	5.00E–54
11	PvContig2749	1269	2.42E–03	O81390 Calcium-dependent protein kinase (*N. tabacum*)	1.00E–153
11	PvContig2764	776	2.83E–02	Q9AR93 Putative calmodulin-related protein (*M. sativa*)	4.00E–50
11	PvContig2825	1172	2.42E–03	Q8H0B4 Wound-induced protein kinase (*N. benthamiana*)	4.00E–99
11	PvContig2949	1350	1.35E–03	P43293 Probable serine / threonine-protein kinase NAK (*A. thaliana*)	1.00E–137
14	PvContig2577	988	5.52E–04	Q8SBC4 Protein phosphatase 2C (*A. thaliana*)	1.00E–100
15	PvContig1792	717	2.35E–02	Q9SXT5 Rab-type small GTP-binding protein (*C. arietinum*)	3.00E–98
17	PvContig1255	572	2.35E–02	O22443 Seed coat peroxidase precursor (*G. max*)	3.00E–32
17	PvContig1784	853	2.35E–02	O80822 Peroxidase 25 precursor (*A. thaliana*)	1.00E–111
17	PvContig1853	1060	3.60E–03	O23961 Peroxidase precursor (*G. max*)	1.00E–133
17	PvContig2404	807	3.60E–03	Q9XFL3 Peroxidase 1 (*P. vulgaris*)	8.00E–87
17	PvContig2938	1167	1.35E–03	O23961 Peroxidase precursor (*G. max*)	1.00E–154
18	PvContig1254	762	2.35E–02	Q93XA0 TGA-type basic leucine zipper protein TGA2.2 (*P. vulgaris*)	1.00E–122
18	PvContig1732	727	2.35E–02	Q9SJR6 Putative ABC transporter (*A. thaliana*)	1.00E–114
18	PvContig1808	573	2.35E–02	Q5W274 PDR-like ABC transporter (*N. tabacum*)	1.00E–63
18	PvContig1821	584	2.35E–02	Q9C6R7 ABC transporter, putative (*A. thaliana*)	5.00E–43
18	PvContig2406	1225	5.52E–04	Q9M9E1 Putative ABC transporter (*A. thaliana*)	1.00E–133

Table 5. (*continued*)

Group number	Contig name	Contig length	Probability of over-representation in P-starved roots	Top Uniref100 TLASTX B	E-value
31	PvContig1813	733	2.35E–02	Q4JL82 MYB transcription factor MYB48-2 (*A. thaliana*)	2.00E–20
31	PvContig1742	769	2.35E–02	Q4JL82 MYB transcription factor MYB48-2 (*A. thaliana*)	1.00E–07
42	PvContig655	821	2.35E–02	Q6R7N3 Putative WRKY transcription factor 30 (*Vitis aestivalis*)	7.00E–43
42	PvContig2572	849	1.28E–02	Q3LHK9 Double WRKY type transfactor (*S. tuberosum*)	3.00E–46
42	PvContig2941	1455	7.88E–05	Q2PJR9 WRKY78 (*G. max*)	1.00E–128
56	PvContig1790	645	2.35E–02	Q9XFX0 Cytochrome P450 monooxygenase (*C. arietinum*)	2.00E–76
56	PvContig2816	806	8.46E–05	Q8S4C0 Isoflavone synthase (*P. lobata*)	1.00E–111
56	PvContig3066	1671	7.15E–04	P93147 Cytochrome P450 81E1 (*G. echinata*)	1.00E–155
57	PvContig1789	999	2.35E–02	Q6L3K1 Putative cinnamoyl-CoA reductase (*S. demissum*)	1.00E–125
57	PvContig1801	912	2.35E–02	O65152 Putative cinnamyl alcohol dehydrogenase (*M. domestica*)	1.00E–115
62	PvContig3189	1151	3.05E–10	No BLASTX Hit	
63	PvContig3106	1268	3.24E–05	P34921 Glyceraldehyde-3-phosphate dehydrogenase (*D. caryophyllus*)	1.00E–156
74	PvContig1828	718	2.35E–02	Q8H8C8 Putative AMP-binding protein (*O. sativa*)	1 / E–92
77	PvContig1718	705	2.35E–02	Q9C938 Putative oxidoreductase (*A. thaliana*)	1.00E–56
77	PvContig1767	673	2.35E–02	Q84L58 1-aminocyclopropane-1-carboxylic acid oxidase (*C. arietinum*)	1.00E–113
77	PvContig2400	846	3.60E–03	Q9C939 Putative oxidoreductase (*A. thaliana*)	1.00E–59
77	PvContig2407	914	5.52E–04	Q9C939 Putative oxidoreductase (*A. thaliana*)	3.00E–64
79	PvContig1737	929	2.35E–02	Q6L4C8 Putative zinc finger protein (*O. sativa*)	6.00E–31
84	PvContig1835	589	2.35E–02	Q700B1 Non-cyanogenic β-glucosidase (*C. arietinum*)	1.00E–40
179	PvContig1804	690	2.35E–02	P25986 Pathogenesis-related protein 2 (*P. vulgaris*)	1.00E–81
179	PvContig2421	626	5.52E–04	P25986 Pathogenesis-related protein 2 (*P. vulgaris*)	5.00E–78
179	PvContig2964	727	1.98E–06	Q41125 Proline-rich 14-kDa protein (*P. vulgaris*)	9.00E–52
179	PvContig3247	795	1.07E–41	P25985 Pathogenesis-related protein 1 (*P. vulgaris*)	2.00E–82
257	PvContig2414	726	1.28E–02	Q8H6U3 Cyclic nucleotide-gated channel A (*P. vulgaris*)	1.00E–74
286	PvContig3084	695	1.08E–02	Q9FNV7 Auxin-repressed protein (*R. pseudoacacia*)	2.00E–34

Discussion

The main goal of our study was to identify candidate genes from *P. vulgaris* that may be important in adaptation to P-stress. Our analysis was initiated with 2883 contigs identified in P-stressed roots of *P. vulgaris*. Using statistical and cluster analyses of EST library composition, we identified 247 candidate contigs that were statistically over-represented in P-stressed roots. Given the fact that we only had data from P-stressed roots it was not possible to ascertain whether these contigs were statistically over-represented generally in roots or specifically in response to P starvation. Therefore, we postulated that analysis of contigs that are

statistically over-represented in other species, particularly legumes, may provide insight in to *P. vulgaris* genes that respond specifically to P-stress. Using this approach we have identified 52 potential P-stress candidate genes for future research (Table 5). These genes may have universal importance in plant adaptation to P-stress.

The genes over-represented in P starvation in four or five plant species (Table 4) belong to various functional categories. Experimental evidence supports the relevant role of some of the genes identified in plant physiological adaptation to cope with P starvation. For example, several phosphate transporter genes cloned and characterised nearly a decade ago are transcriptionally regulated depending on external P availability (reviewed by Raghothama 1999; Smith 2001). A comprehensive transcriptional analysis of P-stressed *Arabidopsis* (Misson *et al*. 2005) revealed that several genes, including members of the Pht1 family of P transporters, ATP-binding cassette (ABC) transporters, peroxidases, transcription factors, organic acid synthesis as well as genes involved in sulfolipid synthesis are induced during P starvation (Misson *et al*. 2005). In our study, several genes involved in P acquisition or transport and mobilisation, such as phosphate transporters, aquaporins, ABC transporters, and phosphatases, were identified as candidate genes for universal response to P stress (Tables 4, 5). Experimental evidence from both *L. albus* and *P. vulgaris* support these results. Macroarray analysis in *L. albus* showed that several transporter, organic acid synthesis, and purple acid phosphatase genes are induced in P-starved roots (Uhde-Stone *et al.* 2003). RNA-blot experiments have shown that aquaporin gene expression is induced in P-starved roots of *P. vulgaris* relative to nodules and roots from normal plants and phosphatase transcripts were only detected in P-starved roots (Ramírez *et al*. 2005).

Accumulation of active oxygen species resulting in oxidative stress is common to several abiotic stresses including deficiency of nutritional elements in several plant species (Bartoz 1997). After prolonged P starvation, *P. vulgaris* plants show several symptoms of oxidative stress such as increased lipid peroxidation and hydrogen peroxide concentrations, and higher catalase and peroxidase activities in P-deficient roots than control roots (Juszczuk *et al*. 2001). In agreement with these reports, we find that peroxidase genes are over-represented as P starvation contigs in *P. vulgaris*, as well as in the other four plant species (Tables 4, 5). In addition, Table 4 shows genes that are often induced in response to elicitors, microbial attack, or under abiotic stress, which may be relevant for plant responses to P starvation, such as pathogenesis-related (PR) proteins and cytochrome P450s.

Notably chlorophyll *a* / *b*-binding protein was over-represented in four of the five species analysed (Table 4). This observation would be consistent with the dark-green leaf coloration that frequently accompanies P stress (Reuter *et al.* 1997). Although overall growth is eventually reduced in P-stressed plants, new leaves are continually generated at the expense of older leaves. These newly generated leaves synthesise the light-capture apparatus; thus, chlorophyll *a* / *b*-binding genes remain highly expressed (Utriainen and Holopainen 2001).

Genes with possible function in regulation or signal transduction pathways, such as protein kinases, zinc finger proteins and transcription factors, are also over-represented in four or five of the datasets analysed (Tables 4, 5). Transcription factors and signal transduction genes that display enhanced expression during P-deprivation are likely to play important roles in other stress conditions. Our analysis, as well as that of Misson *et al*. (2005), identified both WRKY and MYB transcription factors. Rubio *et al*. (2001) noted that a conserved MYB TF is involved in P-starvation signalling both in plants (*Arabidopsis*) and algae (*Chlamydomonas*).

Soil P limitation is a primary effector of root architecture, which refers to the complexity of root spatial configurations that arise in response to soil conditions (López-Bucio *et al*. 2003). Elegant experiments with common bean coupled to simulation modelling have shown that phenotypic adaptations to P deficiency involve changes in root architecture that facilitate acquisition of P from the topsoil (Ge *et al*. 2000; Lynch and Brown 2001). Although changes in endogenous concentrations of growth hormones such as ethylene and auxins have been proposed to mediate modifications in root architecture (López-Bucio *et al*. 2003), the signal transduction pathways or regulatory cascades for this complex plant response remain unknown. Genes such as those reported here (Tables 4, 5) that belong to the functional category of regulation / signal transduction may be relevant for regulating universal plant responses to P deficiency, such as modification of root architecture. Interestingly, a NAK gene containing an miRNA binding site is among the genes noted in Table 5. Recently miRNAs have been implicated in the P-starvation response of *Arabidopsis* (Miura *et al*. 2005; Chiou *et al*. 2006).

Experiments are currently underway to confirm the relevant role of candidate genes for *P. vulgaris* (Tables 4, 5) in the response and adaptation to P starvation. These experiments will compare the transcript profile of roots and nodules from P-deficient bean plants with control plants using approaches such as macroarrays, RNA-blot analysis and real-time quantitative PCR. Preliminary studies of *P. vulgaris* P-response candidate genes by RT–PCR have shown a WRKY and peroxidase expression up-regulated by P-stress (M Ramirez, CP Vance unpubl. data).

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Anexo VII.2

Phosphorus Stress in Common Bean: Root Transcript and Metabolic Responses[1][W][OA]

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Phosphorus (P) is an essential element for plant growth. Crop production of common bean (*Phaseolus vulgaris*), the most important legume for human consumption, is often limited by low P in the soil. Functional genomics were used to investigate global gene expression and metabolic responses of bean plants grown under P-deficient and P-sufficient conditions. P-deficient plants showed enhanced root to shoot ratio accompanied by reduced leaf area and net photosynthesis rates. Transcript profiling was performed through hybridization of nylon filter arrays spotted with cDNAs of 2,212 unigenes from a P deficiency root cDNA library. A total of 126 genes, representing different functional categories, showed significant differential expression in response to P: 62% of these were induced in P-deficient roots. A set of 372 bean transcription factor (TF) genes, coding for proteins with Inter-Pro domains characteristic or diagnostic for TF, were identified from The Institute of Genomic Research/Dana Farber Cancer Institute Common Bean Gene Index. Using real-time reverse transcription-polymerase chain reaction analysis, 17 TF genes were differentially expressed in P-deficient roots; four TF genes, including MYB TFs, were induced. Nonbiased metabolite profiling was used to assess the degree to which changes in gene expression in P-deficient roots affect overall metabolism. Stress-related metabolites such as polyols accumulated in P-deficient roots as well as sugars, which are known to be essential for P stress gene induction. Candidate genes have been identified that may contribute to root adaptation to P deficiency and be useful for improvement of common bean.

Common beans (*Phaseolus vulgaris*) are the world's most important grain legume for direct human consumption; they comprise 50% of the grain legumes consumed worldwide (Broughton et al., 2003; Graham et al., 2003). In several countries of Central and South America, beans are staple crops serving as the primary source of protein in the diet. Environmental factors, such as low soil nitrogen (N) and phosphorus (P) levels, and acid soil conditions are important constraints for bean production in most of the areas where this crop is grown (Graham et al., 2003). In bean, symbiotic N fixation rates, seed protein level, and tolerance to P deficiency are low in comparison to other legumes (Broughton et al., 2003).

P is an essential element required for plant growth and development. Besides N, P is the most limiting nutrient for plant growth, and it is a common limiting factor for crop production in arable soils. Plants have evolved general strategies for P acquisition and use in limiting environments that include: mycorrhizal symbioses, decreased growth rate, remobilization of internal inorganic phosphate (P_i), modification of carbon (C) metabolism bypassing P-requiring steps, increased production and secretion of phosphatases,

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exudation of organic acids, modification of root architecture, expansion of root surface area, and enhanced expression of P_i transporters (for review, see Raghothama, 1999; Smith, 2001; Vance et al., 2003; Plaxton, 2004).

In contrast to disease-resistance traits, where resistance may be due to a single dominant or recessive gene, enhancing tolerance to P stress requires multiple genes and involves several different mechanisms. In recent years, macro/microarray technologies have provided valuable information on global changes in gene expression in response to P starvation in several plant species and organs, including white lupin (*Lupinus albus*) proteoid roots (Uhde-Stone et al., 2003), rice (*Oryza sativa*) leaves and roots (Wasaki et al., 2003, 2006), and Arabidopsis (*Arabidopsis thaliana*) roots, shoots, and leaves (Hammond et al., 2003; Wu et al., 2003; Misson et al., 2005; Müller et al., 2007).

Although macro/microarray studies have identified genes differentially regulated by P starvation, little is known about the regulation of gene expression changes. Transcription factors (TFs) are master control proteins in all living cells, regulating gene expression in response to different stimuli (Riechmann, 2002; Czechowski et al., 2004). Chen et al. (2002) reported that Arabidopsis TF gene expression is regulated in a cell type- or tissue-specific manner and in response to specific environmental biotic and abiotic stresses. Müller et al. (2007) reported that specific TFs are induced in Arabidopsis P-starved leaves. These studies have opened new possibilities to elucidate the sensing, signaling, and regulatory pathways of the P deficiency response in plants.

Despite the agronomic importance of beans, there is little information on global gene expression of bean tissues in response to P deficiency. In previous work, we attempted to identify candidate P stress-induced genes in beans using an in silico approach that clustered bean ESTs with previously identified P stress-induced genes across three other legume species and Arabidopsis (Graham et al., 2006). Here, we undertook a three-step approach to identify genes important to P deficiency in common bean. First, macroarray technology was used for transcript profiling of P-deficient bean roots with the aim of identifying those genes, gene networks, and signaling pathways that are important for the plant response to P deficiency. Second, we identified bean TFs and used quantitative reverse transcription (RT)-PCR to assess TF gene expression in P-deficient bean roots, with the aim of identifying TFs that regulate the differential expression of genes during P stress. Third, we performed nonbiased metabolite profiling of bean roots using gas chromatography coupled to mass spectrometry (GC-MS) to correlate metabolic differentiation orchestrated by global changes in gene transcription as response to P starvation. The overall goal of this research is to identify candidate genes that may be useful to bean improvement and that will contribute to understanding common bean adaptation to P deficiency.

RESULTS

Phenotypic Characterization

The long-term P deficiency treatment used in this work consisted of growing common bean plants in pots under controlled environments for 3 weeks using 200-fold lower phosphate concentration as compared to P-sufficient (+P) control plants. Control plants accumulated higher concentrations of soluble P_i. P_i content in +P leaves was 2.6- and 13-fold higher than in +P stems and +P roots, respectively (Fig. 1A). Compared to +P plants, a drastic reduction (2–23-fold lower) in P_i content was observed in plants grown under P-deficient conditions (Fig. 1A). P_i content in P-deficient plants was similar in leaf, stem, and root tissues (Fig. 1A). Typical P stress responses were observed (Raghothama, 1999; Gilbert et al., 2000; Ma et al., 2003), including a 4-fold reduction in leaf area and 1.5-fold higher dry weight root to shoot ratio (Fig. 1, B and C). The latter response was due to arrested shoot growth and proliferation of lateral roots and root hairs of P-deficient plants.

Content of photosynthetic pigments such as chlorophyll *a* and *b* and carotenes was similar in plants under −P and +P treatments (data not shown). However, P-deficient plants showed significant inhibition of net photosynthetic rate (P_n) regardless of internal CO_2 (C_i) concentration (Fig. 1D). In contrast, P-deficient plants showed 50% lower P_n at ambient CO_2 concentration (350 μmol mol^{-1}), reflecting lower carboxylation efficiency. In addition, P-stressed plants showed 60% of the maximum P_n of +P plants, which is consistent with changes associated with increasingly larger limitations of P_n by Rubisco and ribulose 1,5-bisphosphate regeneration as leaf P_i declines (Fig. 1D). However, stomatal conductance and resistance was not altered in P-deficient plants (data not shown).

Macroarray Analysis of Root Response to P Deficiency

Macroarray analyses were performed to evaluate gene expression from P-deficient roots of bean plants as compared to control P-sufficient roots. Nylon filter arrays were spotted with ESTs that represented a 2,212 bean unigene set consisting of 1,194 singletons and 1,018 contigs derived from the −P roots cDNA library from bean 'Negro Jamapa 81' previously reported (Ramírez et al., 2005; Graham et al., 2006).

Total RNA was isolated from plants grown under similar conditions as described for each treatment (−P and +P). Ten nylon filter arrays were hybridized with first-strand cDNA synthesized from four independent sources of total RNA. From the 10 hybridizations, six replicates with high determination coefficients ($r^2 \geq 0.8$) were chosen for analysis of differential gene expression. A total of 126 cDNAs showed significant ($P \leq 0.05$) differential expression (Tables I and II).

Tables I and II list the genes that were significantly induced or repressed, respectively, in P-deficient roots. To aid in annotation, cDNAs were assigned to tentative

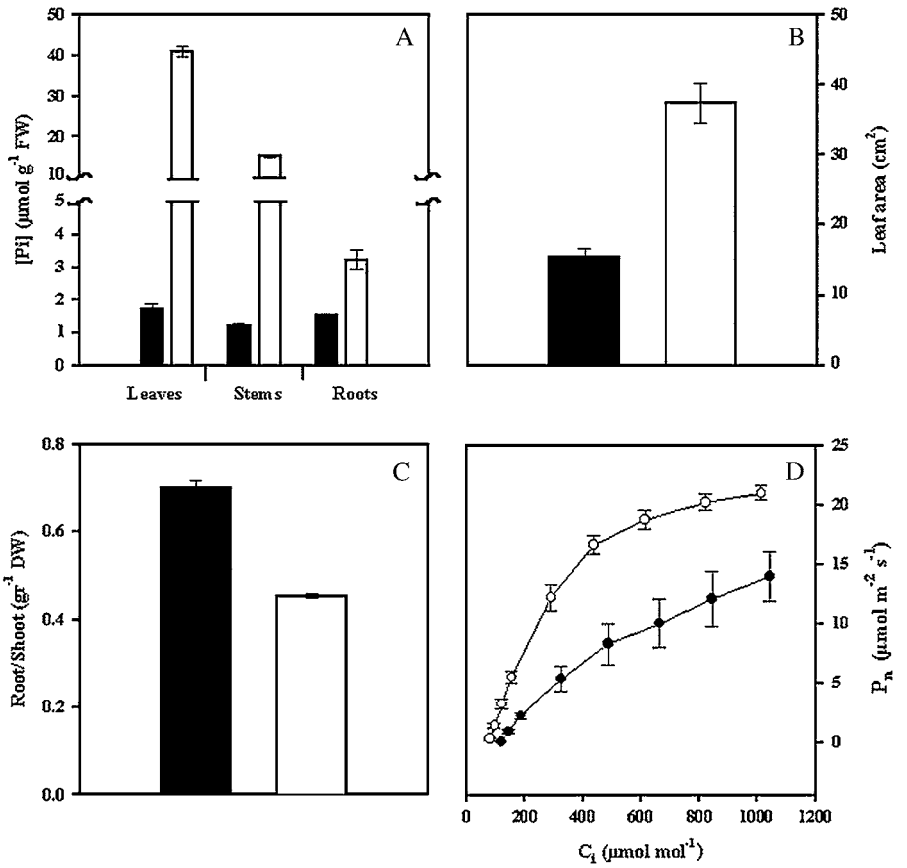

Figure 1. Effect of P deficiency on common bean. A, Soluble P_i content in different plant organs. B, Leaf area from fully expanded leaves. C, Root to shoot dry weight ratio. D, P_n rate as a function of changing C_i. Plants were grown for 3 weeks under P-deficient (black bars or circles) or in P-sufficient conditions (white bars or circles). Values are mean ± SE from 12 determinations: three independent experiments with four replicates per experiment.

consensus sequences (TCs; Institute of Genomic Research [TIGR]/Dana Farber Cancer Institute [DFCI] Common Bean Gene Index, v. 1.0) when possible. The TC or EST sequences were then compared (BLASTX, $E < 10–4$; Altschul et al., 1997) to the Uniprot protein database (Apweiler et al., 2004) to assign putative function. Based on information available in the literature, sequences were then assigned to functional categories.

Table I shows the genes (78) that were induced 2-fold or more in P-deficient roots, classified in nine functional categories. The ''unknown function'' category included those genes with similarity to hypothetical proteins with unknown function and those for which no BLAST hit was found. The two most abundant functional categories, accounting for 23% of genes each, were the regulation/signal transduction category and those coding for genes that participate in secondary metabolism pathways and/or are related to several stress/defense plant responses. Ten genes (13%) were classified as membrane proteins or proteins that participate in transport, both extracellular and intracellular. Six genes (8%) were classified in cell structure, cell cycle, or developmental functions. Nineteen genes (24%) were classified in different metabolic pathways: P_i cycling, C and N metabolism, amino acid/protein synthesis or degradation, and lipid metabolism. Finally, 9% of genes had no known function.

Table II lists the functional classification of the genes (48) that were repressed in −P roots as compared to control roots. The most abundant category was the amino acid/protein metabolism with 11 genes (23%). Only five genes participating in metabolic C/N pathways were identified (10%), and no genes involved in P_i cycling were identified. Nine (19%) and seven (15%) genes were classified in the transport/membrane protein and cell structure/cell cycle/development categories, respectively. Only 8% and 6% of the repressed genes participate in regulation/signal transduction and secondary metabolism/defense pathways, respectively.

It was evident that a number of genes from within a single functional category could either be induced (Table I) or repressed (Table II). We found that 10 P deficiency-induced genes identified by the macroarray analysis had been previously proposed by Graham et al. (2006) as candidate P stress-induced genes in bean (Table I). Graham et al. (2006) identified candidate P stress-induced genes of bean by statistical analysis of contigs overrepresented with ESTs from P-stressed tissues and by clustering candidates with P stress-induced genes identified from a variety of plant species, including Arabidopsis, lupin, soybean, *Medicago truncatula*, and bean. As expected, none of the −P-repressed genes identified by macroarrays (Table

Table I. *Genes induced in roots of P-deficient plants identified by macroarray analysis*

Functional categories are in bold. TC No., Tentative consensus sequence assignment (TIGR/DFCI Common Bean Gene Index, version 1.0); blank cells correspond to singletons with no TC number assigned.

EST Identification	TC No.	GenBank Accession No. of EST	Annotation	BLASTX E-Value	Expression Ratio −P to +P	*P*-Value
P_i cycling						
RTS_113_H08		EH791066	(Q84MA2) Type I inositol-1,4,5-trisphosphate 5-phosphatase	4.00E-10	2.59	9.8E-04
RTS_145_F08	TC1447	CV544205	(Q6J5M7) Purple acid phosphatase 1	1.00E-100	2.10	2.2E-02
RTS_105_G04		CV541472	(Q9LDA7) Protein phosphatase type 2C	1.00E-65	2.12	4.8E-02
C/N metabolism						
RTS_101_F08	TC1804	CV541174	(Q96558) UDP-Glc-6-dehydrogenase	1.00E-65	3.63	1.7E-07
RTS_122_G12	TC733	CV542619	(Q8S532) Cytosolic aldehyde dehydrogenase RF2C	3.00E-48	3.48	7.1E-04
RTS_104_C06	TC1280	CV541371	(P39866) Nitrate reductase	1.00E-122	2.65	2.5E-04
RTS_117_F10		CV542239	(Q75GR9) Inosine-uridine preferring nucleoside hydrolase	3.00E-78	2.28	3.8E-03
Amino acid/protein metabolism						
RTS_104_E06		CV541391	(Q8LSY7) Phosphoribosyltransferase	3.00E-19	6.18	3.60E-05
RTS_117_A02	(TC1704)[a]	EH791074	(Q8LJW0) 40S Ribosomal S4 protein	1.00E-126	2.43	7.30E-04
RTS_108_F01	TC492	CV541666	(Q7DLS1) Proteasome subunit-β type	1.00E-135	2.41	3.20E-03
RTS_120_H04	TC486	CV542476	(P29144) Tripeptidyl-peptidase 2	2.00E-14	2.39	5.00E-02
RTS_101_D04	TC250	CV541149	(Q8LKU3) 60S Ribosomal protein	1.00E-103	2.34	6.80E-04
RTS_117_A07	(TC1704)[a]	EH792674	(Q8LJW0) 40S Ribosomal S4 protein	1.00E-126	2.30	3.30E-04
RTS_135_E01	TC112	CV543464	(Q41119) Cyclophilin	1.00E-88	2.25	1.70E-03
RTS_123_H06		CV542691	(Q9ZNS5) Ribosomal protein S28	4.00E-19	2.20	5.20E-03
RTS_121_C06	TC1703	CV542506	(Q5YJR8) 60S Acidic ribosomal protein	1.00E-23	2.08	3.80E-03
RTS_101_B01	TC85	CV541126	(Q8W3Y4) *S*-adenosyl-methionine synthetase	0.0	2.00	5.70E-03
Lipid metabolism						
RTS_110_A04	TC1739	CV541751	(Q69JE2) Putative CLB1 protein	0.0	2.51	4.2E-02
RTS_127_F12		CV542902	(Q8LG07) GlcNAc-1-P-transferase	2.00E-79	2.21	1.2E-03
Cell structure/cell cycle/development						
RTS_109_B06		EH792671	(Q9LVI9) Senescence-related dihydroorotate dehydrogenase	1.00E-50	5.11	3.30E-08
RTS_125_D07	TC63	CV542788	(Q944T2) Translationally controlled tumor protein	3.00E-77	3.72	7.60E-04
RTS_127_C08	(TC1617)[a]	EH792676	(Q41125) Pro-rich, 14-kD protein	1.00E-50	2.43	7.10E-05
RTS_137_A05	TC114	CV543578	(Q41707) Extensin class 1 protein precursor	1.00E-134	2.40	9.10E-05
RTS_119_E03	TC254	CV542369	(Q9XHD5) B12D/Leaf senescence protein	4.00E-35	2.21	7.60E-04
RTS_136_B08	TC415	CV543516	(Q09085) Hydroxy-Pro-rich glycoprotein	2.00E-80	2.10	8.80E-04
Stress/defense/secondary metabolism						
RTS_138_E12	TC1903	CV543709	(Q6K1Q5) Glycolipid transfer protein-like	2.00E-82	4.62	2.10E-05
RTS_101_C10	TC280	CV541144	(Q2KTE6) ACC oxidase	1.00E-160	3.59	1.10E-03
RTS_107_E03	TC64	CV541595	(P25985) Pathogenesis-related protein (PvPR1)[b]	2.00E-82	3.00	5.40E-05
RTS_135_E10	TC459	CV543472	(Q9C939) Putative oxidoreductase[b]	1.00E-59	2.63	4.40E-04
RTS_117_E09	TC2562	CV542227	(Q700B1) Noncyanogenic β-glucosidase[b]	9.00E-41	2.54	2.30E-05
RTS_113_H03	TC1260	CV541991	(Q2LAL0) Cyt P450 monooxygenase[b]	1.00E-72	2.52	1.80E-03
RTS_111_F07	TC2443	CV541849	(Q2LAL4) Cyt P450 monooxygenase[b]	1.00E-56	2.49	1.50E-03
RTS_103_E06	TC1890	CV541320	(Q9SWS4) Ripening-related protein/(O65884) MLP protein	1.00E-71	2.39	2.10E-03
RTS_136_E10	TC397	CV543546	(O22443) Seed coat peroxidase precursor[b]	1.00E-103	2.31	3.30E-03
RTS_113_H10		CV541995	(P32292) Indole-3-acetic acid-induced protein ARG2	5.00E-39	2.28	2.60E-03
RTS_119_F09	TC146	CV542386	(O48561) Catalase-4	0.0	2.27	1.30E-04
RTS_102_H03	TC2059	CV541272	(O23961) Peroxidase precursor[b]	1.00E-119	2.26	3.10E-02
RTS_135_B05		CV543440	(Q1SGR7) TIR; Disease resistance protein	1.00E-49	2.18	4.50E-03
RTS_138_A10	TC257	CV543662	(P07218) Phe ammonia-lyase	0.0	2.18	2.80E-03
RTS_108_H02		CV541683	(Q4QTI9) Pathogenesis-related 10 protein	1.00E-15	2.15	2.10E-02
RTS_125_H01		CV542814	(Q1SGA8) 2OG-Fe(II) oxygenase	2.00E-38	2.12	2.90E-02
RTS_118_D09		CV542294	(Q8LJ95) NBS-LRR resistance protein RGH1-like	1.00E-06	2.04	1.40E-02
RTS_105_D07	TC1309	CV541452	(O65152) Cinnamyl alcohol dehydrogenase[b]	1.00E-115	2.04	3.80E-03
Transport/membrane proteins						
RTS_113_A06		CV541934	(Q1SI67) Dynamin central region	1.00E-43	3.96	5.00E-04
RTS_108_A08	TC220	CV541629	(Q506K0) Putative aquaporin[b]	1.00E-164	2.91	2.70E-05
RTS_133_A05	TC2513	CV543276	(O65744) GDP dissociation inhibitor	4.00E-22	2.48	2.80E-02

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

EST Identification	TC No.	GenBank Accession No. of EST	Annotation	BLASTX E-Value	Expression Ratio −P to +P	*P*-Value
RTS_109_H05	TC2647	CV541745	(Q6YZC3) Glc-6-P/phosphate translocator	1.00E-109	2.33	2.30E-02
RTS_108_H06	TC587	CV541687	(Q9MAX8) Epsilon1-COP	1.00E-157	2.23	8.10E-03
RTS_108_F03	TC2564	CV541668	(Q8H4Q9) GTP-binding protein Rab6	5.00E-34	2.21	4.70E-02
RTS_124_F11		CV542748	(Q65CB1) ATP-binding cassette transporter[b]	9.00E-78	2.09	1.10E-03
RTS_119_F08	TC1933	CV542385	(Q9FVE8) Plasma membrane Ca^{2+}-ATPase	1.00E-150	2.05	3.80E-02
RTS_136_H04		CV543570	(Q6K5Y4) Putative UDP-GlcNAc transporter	8.00E-63	2.05	4.10E-02
RTS_103_G05	TC2845	CV541336	(Q1T029) Sugar transporter superfamily	2.00E-75	2.00	1.30E-02
Regulation/signal transduction						
RTS_121_B11	TC189	CV542500	(Q9LEB4) RNA Binding Protein 45	1.00E-122	3.21	8.40E-04
RTS_104_H09		CV541419	(Q41109) Regulator of MAT2	1.00E-23	3.11	1.50E-04
RTS_119_D05	TC1707	CV542360	(Q39892) Nucleosome assembly protein 1	1.00E-122	3.09	2.10E-07
RTS_104_D03	TC1670	CV541379	(Q1SGW5) ZIM[c]	1.00E-24	2.76	4.70E-05
RTS_101_E04		CV541158	(Q1RVC8) HMG-I and HMG-Y, DNA-binding	1.00E-22	2.44	2.20E-03
RTS_138_A05		CV543658	(O65573) PRL1-associated protein	6.00E-34	2.39	1.70E-02
RTS_128_D02		CV542946	(Q8GXM5) RING-H2 finger protein RHB1a	4.00E-25	2.37	1.00E-02
RTS_142_A06	TC778	CV543967	(Q9SK39) Putative steroid-binding protein 3	2.00E-40	2.34	6.20E-03
RTS_102_F11		CV541259	(Q8LCS8) NTGP5	4.00E-14	2.30	2.50E-03
RTS_108_H08		CV541689	(Q1T5G4) Zinc finger, CCCH-type	1.00E-29	2.20	2.70E-02
RTS_103_H07	TC2434	CV541346	(Q5Z6C2) Putative mlo2 protein	4.00E-63	2.17	2.50E-02
RTS_101_B04	TC2165	CV541128	(Q8H1A5) DEAD box RNA helicase	0.0	2.16	2.20E-02
RTS_101_F12	TC1622	CV541178	(Q3HLY8) U-box protein	1.00E-109	2.09	1.70E-02
RTS_104_B03	TC348	CV541358	(Q5MJ53) AT-rich element binding factor 3	3.00E-91	2.08	2.90E-03
RTS_110_H08	TC221	CV541801	(Q93XA5) Homeodomain Leu zipper protein	1.00E-119	2.08	2.80E-03
RTS_119_B12	TC100	CV542349	(Q7F8L1) Histone H3	2.00E-65	2.05	1.40E-03
RTS_137_H03	TC165	CV543649	(Q93VL8) Calmodulin	1.00E-75	2.00	3.20E-02
RTS_123_D08	TC1622	CV542656	(Q3HLY8) U-box protein	1.00E-109	2.00	3.70E-02
Unknown						
RTS_117_G02	TC1992	CV542243	(Q1SEK2) Hypothetical protein	1.00E-36	7.15	5.20E-05
RTS_113_E03		CV541966	No BLAST hit <10–4	–	4.89	2.90E-05
RTS_121_D02		CV542512	No BLAST hit <10–4	–	2.63	4.70E-04
RTS_123_C04		EH792675	No BLAST hit <10–4	–	2.46	7.50E-04
RTS_119_F10		EH791078	(Q8W4E6) Hypothetical protein	1.00E-90	2.22	1.40E-03
RTS_104_C07		CV541372	No BLAST hit <10–4	–	2.06	3.30E-02
RTS_123_D12		CV542660	No BLAST hit <10–4	–	2.00	5.80E-03

[a]BLAST analysis of this new gene sequence revealed an overlap with the indicated TC from the TIGR/DFCI Common Bean Gene Index. [b]Genes reported as bean candidate P stress-induced genes through clustering analysis across five or four plant species by Graham et al. (2006). [c]Annotation according to TF genes identified in this work (Table III; supplemental data).

II) were included in the Graham et al. (2006) analysis, which only evaluated induced genes.

Expression Analyses of Selected Genes by RT-PCR

Nine ESTs selected from both Tables I and II (18 total) were chosen to assess whether macroarray expression data could be confirmed by an alternate method. We performed semiquantitative RT-PCR on ESTs representing at least four functional categories designated in Tables I and II. As shown in Figure 2, all 18 genes tested for expression by RT-PCR gave results confirming their expression obtained with macroarray experiments. From the P deficiency stress-induced genes, UDP-Glc-6-dehydrogenase, senescence-related dihydroorotate dehydrogenase, glycolipid transfer protein, and hypothetical protein were the most highly induced genes in their particular categories, as measured by macroarrays. These genes showed enhanced expression by RT-PCR (Fig. 2A). Likewise, from the P deficiency-repressed genes in Table II, isocitrate dehydrogenase, SAM-decarboxylase, multidrug resistance protein, and caffeine-induced death protein were among the most highly repressed genes detected by macroarray analysis (Fig. 2B), and these genes showed reduced expression in P deficient as compared to P sufficient when evaluated by RT-PCR.

TF Transcript Profiling by Real-Time RT-PCR

The TIGR/DFCI Common Bean Gene Index contains 9,484 total unigenes (2,906 TCs and 6,578 singletons) comprised of 21,290 input EST sequences. The first step in our work was to define the set of bean EST/TC sequences in the TIGR/DFCI Common Bean Gene Index (www.tigr.org; http://compbio.dfci.harvard.edu/tgi/plant.html) coding for proteins with InterPro domains diagnostic or characteristic of TF genes. A total of 372 sequences, corresponding to 4% of the bean unigene set, was identified using 41 of the preselected

Table II. *Genes repressed in roots of P-deficient plants identified by macroarray analysis*

Functional categories are in bold. TC No., Tentative consensus sequence assignment (TIGR/DFCI Common Bean Gene Index, version 1.0); blank cells correspond to singletons with no TC number assigned.

EST Identification	TC No.	GenBank Accession No. of EST	Annotation	BLASTX E-Value	Expression Ratio +P/−P[a]	*P*-Value
C/N metabolism						
RTS_112_G12	TC1864	CV541923	(Q40345) Isocitrate dehydrogenase (NADP)	1.00E-58	−3.36	2.97E-02
RTS_114_F03		CV542030	(Q9C9W6) Trehalose-6-P synthase	2.00E-86	−2.57	2.79E-02
RTS_140_E03	TC2520	CV543865	(Q9SEK4) Succinic semialdehyde dehydrogenase	1.00E-138	−2.25	1,45E-02
RTS_141_C04	TC851	CV543912	(Q8LBR3) Alcohol dehydrogenase	4.00E-56	−2.07	2.92E-02
RTS_114_C03		EH791068	(Q9C9W6) Trehalose-6-P synthase	4.00E-87	−2.02	2.56E-02
Amino acid/protein metabolism						
RTS_140_C05	TC83	CV543847	(Q8W3Y2) *S*-adenosyl-methionine decarboxylase	0.0	−2.82	3.56E-04
RTS_139_D07		CV543777	(Q9SKA3) Phosphoribosylanthranilate transferase	4.00E-55	−2.66	1.22E-02
RTS_141_B12	TC1262	CV543909	(Q6XJF4) 26S Proteasome subunit RPN12	1.00E-114	−2.43	8.06E-03
RTS_131_C09	TC1689	CV543154	(Q9FKC0) 60S Ribosomal protein L13a-4	1.00E-104	−2.39	3.25E-03
RTS_132_D01	TC1613	CV543229	(O81361) 40S Ribosomal protein S8	1.00E-83	−2.34	3.98E-02
RTS_129_D12	TC267	CV543033	(Q9AV87) 60S Ribosomal protein L21	3.00E-84	−2.15	5.54E-03
RTS_141_C11	(TC836)[b]	EH792677	(Q8W538) Ribosomal S15 protein	6.00E-73	−2.15	4.23E-02
RTS_140_D04	TC163	CV543856	(O24322) Cys proteinase precursor	0.0	−2.11	3.68E-02
RTS_140_H12		EH791103	(Q9FY64) Ribosomal protein S15-4	6.00E-77	−2.04	2.33E-02
RTS_112_A10	TC110	CV541878	(Q71EW8) Met synthase	0.0	−2.00	9.52E-03
RTS_123_G08	TC209	CV542684	(P17093) 40S Ribosomal protein S11	3.00E-76	−2.00	1.96E-02
Cell structure/cell cycle/development						
RTS_102_C02	TC184	CV541220	(Q2PK12) Actin depolymerizing factor	6.00E-68	−2.81	4.49E-04
RTS_143_D10		CV544078	(Q6ZH89) Caffeine-induced death protein 1	5.00E-43	−2.76	1.55E-03
RTS_139_D02	TC1176	CV543772	(O80452) AMP deaminase	3.00E-29	−2.70	7.85E-03
RTS_112_C11		EH791061	(Q41125) Pro-rich 14-kD protein	1.00E-10	−2.59	9.88E-03
RTS_114_F01	(TC1723)[b]	EH792673	(O04300) α-1,4-Glucan-protein synthase	0.0	−2.34	1.21E-02
RTS_129_G01	TC697	CV543049	(P93273) Fruit development protein (PAFD103)	2.00E-16	−2.33	2.94E-02
RTS_115_A08	(TC1617)[b]	EH792678	(Q41125) Pro-rich 14-kD protein	1.00E-50	−2.04	1.47E-02
Stress/defense/secondary metabolism						
RTS_105_B12	(TC2799)[b]	EH791098	(Q9LJX0) Multidrug resistance protein 11	1.00E-116	−2.76	1.78E-02
RTS_142_B04	TC692	CV543977	(Q9SZB9) Peroxidase 47 precursor	4.00E-83	−2.19	1.50E-02
RTS_139_B06		CV543755	(Q9SPJ5) Dihydroflavonol-4-reductase DFR1	1.00E-86	−2.14	2.73E-02
Transport/membrane proteins						
RTS_120_F09		EH791079	(Q1SG4) TFG-β receptor, type I/II extracellular region	1.00E-62	−5.88	5.25E-03
RTS_132_D07		CV543235	(Q1SEG6) Heavy metal transport/detoxification protein	5.00E-38	−2.48	2.73E-02
RTS_140_E05	TC558	CV543867	(P31167) ADP, ATP carrier protein 1	3.00E-51	−2.43	2.73E-02
RTS_129_C07	TC2875	CV543019	(Q9LX35) Transporter-like protein	1.00E-56	−2.29	1.01E-03
RTS_129_H01	TC1373	CV543060	(Q8H9B7) Putative lipid transfer protein	3.00E-28	−2.25	4.69E-02
RTS_143_E05		CV544083	(Q8RYH0) GPI-anchored protein-like	4.00E-22	−2.25	1.25E-02
RTS_143_E09	TC1952	CV544086	(Q9LF59) Gly/Pro-rich protein	5.00E-38	−2.20	9.54E-05
RTS_114_C05	TC1657	CV542010	(Q9SMK5) Plasma membrane intrinsic polypeptide	2.00E-51	−2.08	6.63E-03
RTS_141_F10	TC2300	CV543942	(Q9FQ21) Putative Hs1pro-1-like receptor	2.00E-56	−2.02	9.57E-03
Regulation/signal transduction						
RTS_140_A08	TC2273	CV543830	(Q1SDP0) Pathogenesis-related transcriptional factor	6.00E-61	−2.76	4.91E-02
RTS_139_D11		CV543780	(Q6ZFY4) BHLH protein-like	1.00E-06	−2.61	1.88E-02
RTS_129_G09	TC829	CV543056	(Q9M9V8) Calcium-dependent protein kinase 1	1.00-38	−2.36	1.60E-02
RTS_142_D10	(TC1606)[b]	EH791104	(Q9FNV7) Auxin-repressed protein	4.00E-35	−2.23	9.36E-03
Unknown						
RTS_114_E05		CV542025	No BLAST Hit <10–4	–	−2.91	3.43E-02
RTS_112_A12		EH791058	No BLAST Hit <10–4	–	−2.88	3.59E-02
RTS_131_E06		EH791092	Hypothetical protein	2.00E-21	−2.48	2.11E-03
RTS_129_B07		EH795233	No BLAST Hit <10–4	–	−2.43	1.13E-02
RTS_132_A02		EH791093	Hypothetical protein	3.00E-75	−2.29	4.89E-02
RTS_112_C10		EH792672	(Q60EX8) Hypothetical protein	4.00E-51	−2.13	3.64E-02
RTS_122_G01		CV542612	No BLAST Hit <10–4	–	−2.12	1.23E-02
RTS_129_H03	TC1470	CV543062	(Q1S1H6) Hypothetical protein	4.00E-19	−2.03	3.95E-02
RTS_142_E11	TC2851	CV544015	(Q93VT6) Hypothetical protein	1.00E-65	−2.03	1.58E-02

[a]For ratios lower than 1 (genes repressed in P deficiency), the inverse of the ratio was estimated and the sign was changed. [b]BLAST analysis of this new gene sequence revealed an overlap with the indicated TC from the TIGR/DFCI Common Bean Gene Index.

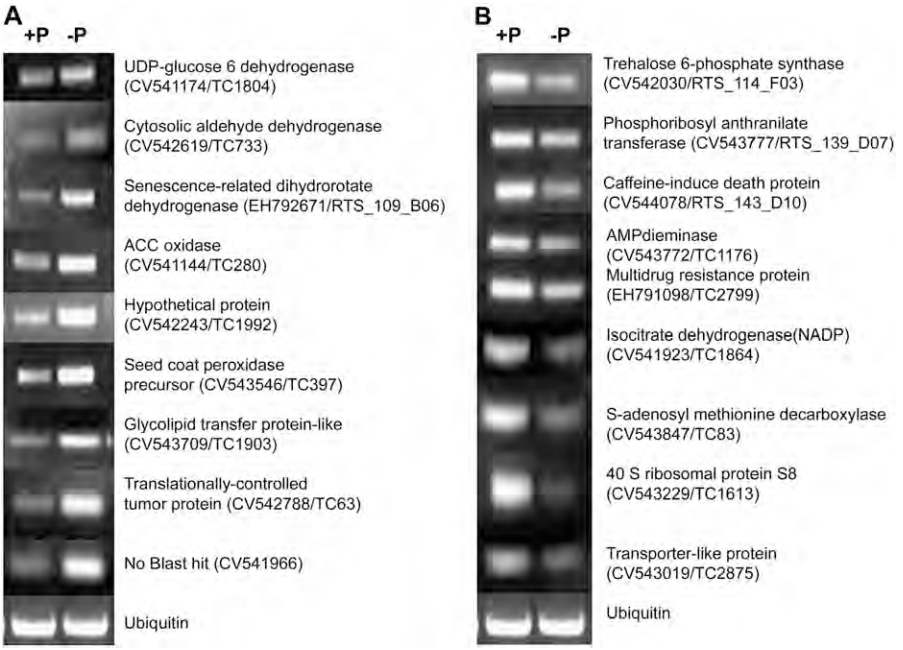

Figure 2. Verification of macroarray results by RT-PCR analysis. Selected genes identified as induced (A) or repressed (B) in P-deficient roots were evaluated. The ubiquitin gene was included as control for uniform RT-PCR conditions (bottom). The primer sequences and reaction conditions used are presented in Table V.

TF diagnostic Inter-Pro domains. This constitutes the whole set of TF genes used for our real-time RT-PCR analyses. Most likely, some of the genes are not true TFs; however, they were included because they contain DNA-binding and other domains that are characteristic of TF proteins. Based on the classification of Arabidopsis TF gene families (Riechmann, 2002; http://range.gsc.riken.ip/rart; http://daft.cbi.pku.edu.cn), bean TF genes were grouped into 47 families (Fig. 3).

Although TF classes in bean were restricted to those identified from cDNA libraries, a general correspondence was found between the most abundant TF families in beans and those from Arabidopsis (Riechmann, 2002), such as the MYB superfamily with 46 gene members (12%), C2H2(Zn) (10%), and AP2/EREBP (8%; Fig. 3). However, in our dataset, we found that CCAAT and bHLH families were equally abundant in our bean TF gene set (Fig. 3), while in Arabidopsis the bHLH

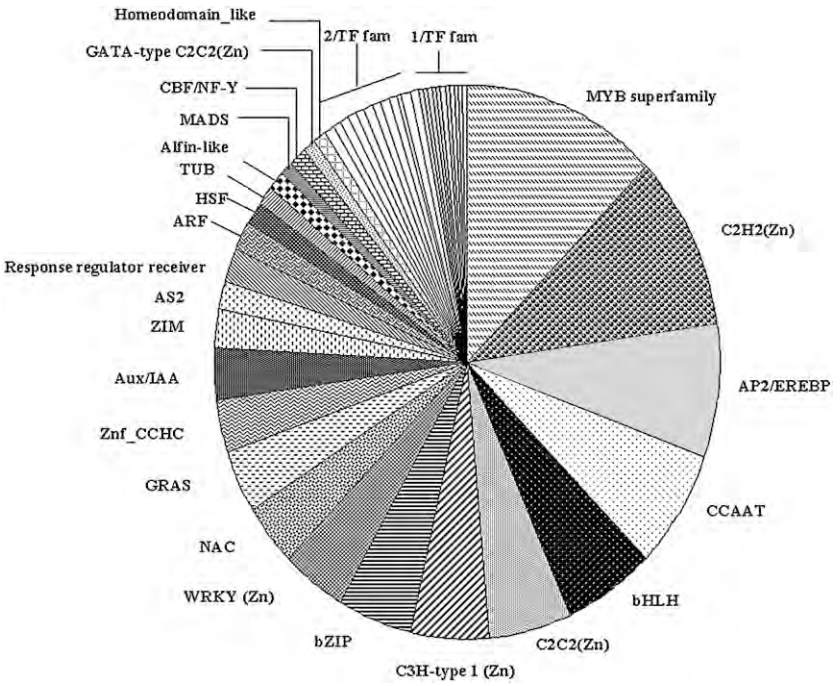

Figure 3. Classification of common bean TF genes in different families. The TF genes (372) were grouped in 47 different families with different Inter-Pro domains according to TF gene families reported for Arabidopsis (Riechmann, 2002; http://range.gsc.riken.ip/rart; http://daft.cbi.pku.edu.cn). The identity of each TF gene family with three or more members is shown. Twelve gene families with two members each (2/TF fam) are: TAZ, MBF1, ARID, Nin-like, Dof-type C2C2(Zn), S1Fa-like, YABBY C2C2(Zn), BES1, K-box, Histone-like/CBFA_NFYB_topo, Auxin_resp, and Lambda_DNA_bd. Eleven gene families with one member each (1/TF fam) are: FHA, LIM-domain, E2F/DP, Jumonji JmjN, SBP, SHAQKYF_MYB_bd, ZF_HD, SRS, POX, EIL, and Euk_TF_DNA_bd.

family is around 3-fold more abundant than the CCAAT family (Riechmann, 2002). Other families of bean TF genes consisted of between one and 12 genes (Fig. 3).

We performed TF profiling based on real-time RT-PCR to determine differential expression of bean TF genes that might be involved in gene expression response to P deficiency. There were three biological replicates of −P- and +P-treated roots. In each RT-PCR run, the phosphatase gene (TC201) was included as a P-deficient marker. This marker gene, known to be induced in P-deficient roots (Ramírez et al., 2005), showed an average expression ratio −P to +P of 18.48 ($P = 0.005$), confirming the P-deficient status of the roots. From the 372 TF genes, 46 (12%) were differentially expressed ($P \leq 0.05$) in −P-treated roots, 10 were induced, and the rest were repressed in −P roots. Table III shows those TF genes that were induced (four) or repressed (13) 2-fold or more in P-deficient roots. To annotate the P-regulated TFs, the TC sequences were blasted (BLASTX, $E < 10^{-4}$; Altschul et al., 1997) against the Uniprot protein database (Apweiler et al., 2004; Table III).

Most of the TF genes induced in −P roots belong to the MYB superfamily (Table III). The induction of Arabidopsis MYB TF genes in response to different biotic stresses (Chen et al., 2002) and to P starvation (Müller et al., 2007) has been shown previously. It has been demonstrated that the Arabidopsis *PHR1* and *PHR2* genes, which resemble the *PSR1* gene from *Chlamydomonas reinhardtii* and belong to the TF MYB superfamily, are crucial for P starvation signaling (Rubio et al., 2001; Todd et al., 2004). Our BLAST analysis revealed that the deduced translated amino acid sequence of MYB TF TC2883, induced 2-fold in −P roots (Table III), showed 59% amino acid identity to *PHR1* (BLASTX E value = $4.1E^{-39}$). The C2C2(Zn) TF family was the most highly represented among the repressed TF genes, and members from eight other TF gene families were also repressed (2-fold or more) in −P roots (Table III).

Metabolome Analyses

To assess the degree to which changes in plant gene expression in P-deficient bean roots affect overall metabolism, we performed nonbiased metabolite profiling of bean roots using GC-MS. The complete information of the 81 metabolites and mass spectral metabolite tags (MSTs) detected in bean roots subjected to both treatments (−P and +P) is provided as supplemental data.

Table IV shows the retention time index (RI) value and RI SD of those metabolites and MSTs (42) with −P to +P response ratios 1.5-fold or more and those with lower ratios but highly significant ($P \leq 0.05$). The metabolites thus identified were in agreement with previous analyses (Desbrosses et al., 2005), mostly primary metabolites belonging to the compound classes: amino acids, organic acids, polyhydroxy acids, fatty acids, sugars, sugar phosphates, polyols, and other nitrogenous compounds. Most of the metabolites showed a response ratio higher than 1, indicating an increase in P-deficient roots; only eight metabolites were decreased in P-stressed roots (Table IV). Most of the amino acids were increased in P-stressed roots; in addition, the polyols and sugars showed high and significantly different −P to +P response ratios (Table IV).

Quantitative data for the metabolites listed in Table IV were used for independent component analysis

Table III. *TF genes significantly expressed in roots of P-deficient plants identified by real-time RT-PCR*

Data of genes exhibiting ≥2-fold induction or repression expression ratio in roots from P-deficient plants versus +P plants.

GenBank Accession No./TC No.	Annotation	TF Family or Domain	Expression Ratio −P to +P[a]	*P*-Value
Induced in −P				
CV532742	MYB family TF	MYB superfamily	3.19	2.5E-02
CV541354	MYB family TF	MYB superfamily	2.12	5.0E-02
TC2883	Transfactor-like protein	MYB superfamily	2.00	4.4E-02
TC1670	Unknown protein At1g19180	ZIM	2.00	4.9E-02
Repressed in −P				
CV535367	Zinc finger protein	C2H2(Zn)	−3.03	5.0E-02
TC1859	Protein kinase (E6)	C2H2(Zn)	−2.00	5.0E-02
TC1802	GPI-anchored protein	C2H2(Zn)	−2.00	5.0E-02
TC2557	RNA-binding protein	C2H2(Zn)	−2.00	1.0E-02
TC2359	LOB domain protein	AS2	−5.95	1.1E-02
CV535841	LOB domain protein	AS2	−3.26	2.2E-02
CB540443	TF	Alfin-like	−2.19	3.6E-02
CV536700	Ethylene response factor	AP2/EREBP	−2.03	1.1E-02
CV530634	bHLH TF	bHLH	−2.59	5.1E-02
CV530350	YABBY2-like TF	C2C2(Zn)	−2.67	3.0E-02
CB542250	WUSCHEL-related homeobox 4	CCAAT	−2.89	4.0E-03
CB540853	Phosphate starvation response regulator	MYB superfamily	−2.00	5.0E-02
CV535056	NAM-like protein	NAC	−2.00	1.0E-02

[a]Whenever the ratio was lower than 1 (genes repressed in P-deficiency), the inverse of the ratio was estimated and the sign was changed.

Table IV. *Metabolites identified by GC-MS in bean roots from −P- and +P-treated plants*

	RI, Expected	RI, SD	Response Ratio −P to +P[a]
Amino acids			
Gly	1,304.5	−0.17	−3.3
β-Ala	1,424.7	−0.12	**1.4**
Ser	1,252.9	0.09	5.2
Asp	1,420.5	0.07	2.1
Asn	1,665.8	−0.02	20.0
4-Aminobutyric acid	1,526.5	−0.15	**1.9**
Thr	1,290.9	−0.02	**3.3**
Pro	1,298.0	−0.16	1.7
Glu	1,615.4	−0.10	2.1
Gln	1,767.6	−0.05	−5.0
Arg (citrulline)[b]	1,814.4	0.07	2.8
Leu	1,151.0	0.52	3.4
Lys	1,847.3	−0.02	3.5
Phe	1,553.1	0.15	5.0
N compounds			
Putrescine (agmatine)[b]	1,737.2	−0.24	**1.4**
Allantoin	2,067.3	0.29	1.6
Spermidine	2,251.1	−0.22	1.6
Urea	1,235.6	0.06	−5.0
Organic acids			
Oxalic acid	1,116.8	0.90	−2.5
Malonic acid	1,195.0	0.30	−2.5
Succinic acid	1,310.2	0.02	**1.4**
Malic acid	1,477.3	0.11	**−1.1**
2-Methylmalic acid	1,464.3	−0.09	**1.3**
Shikimic acid	1,792.5	0.05	1.6
Polyhydroxy acids			
Erythronic acid-1,4-lactone	1,426.9	0.08	**−1.3**
Galactonic acid-1,4-lactone	1,877.4	−0.31	**2.5**
Polyols			
Threitol	1,485.1	−0.12	**2.1**
Arabitol	1,707.8	−0.19	**3.4**
Myoinositol	2,083.9	−0.27	**3.1**
Sugars			
Xyl	1,651.6	−0.12	**1.9**
Gal	1,874.7	0.16	**2.2**
Fru	1,856.2	−0.24	**2.8**
Fru	1,865.9	−0.23	**3.2**
Man	1,869.0	−0.17	**3.5**
Suc	2,629.6	−0.28	**1.2**
MSTs[c,d]			
[516; 1H-Indole-2,3-dione, 1-(tert-butyldimethylsilyl)-5-isopropyl-, 3-(*O*-methyloxime)]	1,691.2	−0.04	−2.0
[771; α-D-Methylfructofuranoside (4TMS)]	1,760.9	−0.02	1.6
[802; Methylcitric acid (4TMS)]	1,909.57	−0.06	2.2
[926; Galactosylglycerol (6TMS)]	2,297.2	−0.12	**5.9**
[802; Gulose (5TMS)]	2,424.3	0.26	**2.0**
[964; Trehalose (8TMS)]	2,678.4	0.02	7.3
[882; Melibiose (8TMS)]	3,092.9	−0.27	**3.5**

[a]The response ratio of average −P root response compared with average +P root response is listed (*t* test significance of $P \leq 0.05$ is indicated by bold format of the response ratio). For ratios lower than 1, the inverse of the ratio was estimated and the sign was changed. [b]Represents the sum of two or more metabolites. [c]Reference substance not yet available. [d]MSTs are characterized by match factor and mass spectral hit.

(ICA) to identify major differences in metabolite composition in P-deficient and normal roots. ICA of metabolite response ratios of all 81 metabolites in 12 samples from P-deficient roots and 12 samples of P-sufficient roots allowed nonbiased partitioning into two sample groups showing gradual differentiation of individual plants from a P-sufficient metabolite phenotype (Fig. 4). The score plots (Fig. 4) show a clear separation between −P and +P samples, though some overlap in the samples can be seen, which probably indicates a P deficiency but not total P starvation in bean roots.

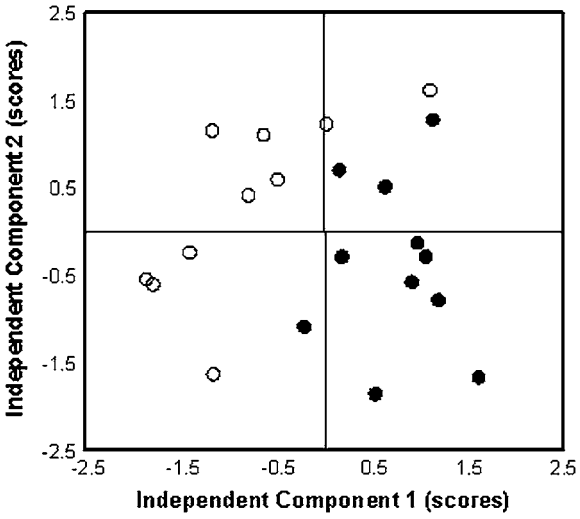

Figure 4. ICA of major metabolic variances in bean roots. Bean plants grown in P-sufficient (white circles) and P-deficient (black circles) conditions were used. Scores analysis demonstrates gradual differentiation of individual plants from a P-sufficient metabolic phenotype.

DISCUSSION

In this report, we have advanced the fundamental understanding of common bean root gene expression and plant adaptation to P deficiency by: (1) identifying differential patterns of gene expression in P-stressed roots through macroarray analysis; (2) identifying 372 TFs and evaluating their expression profile by quantitative RT-PCR; and (3) complementing gene expression analysis with unbiased metabolomic profiling. Transcript expression patterns revealed by hybridization of nylon filter arrays spotted with some 4,000 ESTs from bean −P roots cDNA library (Ramírez et al., 2005) resulted in a total of 126 differentially expressed genes with 2-fold or more induction or repression in −P roots (Tables I and II). In addition, transcript profiling of 372 TF genes identified from the bean gene index (TIGR/DFCI) resulted in 17 differentially expressed (2-fold or more) bean TF genes in −P versus +P bean roots (Table III). Nonbiased metabolite profiling using GC-MS technology led to the identification of 64 metabolites and 17 MSTs from bean roots, 42 of which showed ≥1.5-fold and/or significantly different −P to +P response ratios (Table IV). ICA analysis from the 81 identified metabolites revealed a gradual differentiation of individual plants from a P-sufficient metabolic phenotype (Fig. 4). Our results reveal a suite of responses ranging from changes in growth and development to altered gene expression and metabolic profile that may contribute to adaptation of common bean roots to P deficiency.

An overriding question regarding our macroarray experiments is: are genes designated as having enhanced expression during P stress in actuality responding to low P, or do they show enhanced expression due to root developmental effects? Several pieces of evidence suggest that a great many bean genes are responding to P stress. First, of the 50 TCs listed as induced during P stress in Table I, more than 80% have the majority of ESTs derived from a P stress root library. In fact, 11 of the 50 have 100% of their ESTs derived from the P stress root library. Second, semiquantitative RT-PCR of several P stress-induced genes (Fig. 2) show enhanced expression in P-stressed roots. Furthermore, an in silico statistical analysis of ESTs overrepresented in P stress libraries in legumes and Arabidopsis gene indices, similar to that described by Graham et al. (2006), showed that at least 50% of the TCs in Table I would be predicted to be highly expressed under P stress. Unfortunately, similar statistical methods cannot be applied to singletons or to underexpressed (underrepresented EST) TCs. However, semiquantitative RT-PCR of a number of underexpressed genes in Table II showed that they had reduced expression in P-stressed roots as compared to P-sufficient roots (Fig. 2).

As an initial step in responding to P deficiency, plants must sense that nutrient stress is occurring and transduce appropriate signals into processes that facilitate adaptation. Although the genes affecting P stress signal recognition and transduction in legumes are unknown, studies in Arabidopsis and rice have implicated *MYB* (*PHR1*), *WRKY* (*WRKY75*), and *bHLH* (*OsPTF1*) TFs in the P-signaling process (Rubio et al., 2001; Yi et al., 2005; Devaiah et al., 2007). Recently, the interaction of miRNA 399 with ubiquitin-conjugating enzyme (*UBC*) has also been demonstrated to play a key role in the P stress response of Arabidopsis (Fujii et al., 2005; Miura et al., 2005; Chiou et al., 2006). Our array study as well as those of others (Hammond et al., 2003; Uhde-Stone et al., 2003; Wu et al., 2003; Misson et al., 2005; Morcuende et al., 2007; Müller et al., 2007) have detected a plethora of putative signaling and regulatory genes that could be involved in P stress signaling. We found some 39 genes (Tables I–III) that may contribute to P stress signal transduction/regulation in common bean roots. As in Arabidopsis, we found representatives of *MYB*, *UBC*, and *bHLH* gene families as either up- or down-regulated in expression. We detected three members of the MYB superfamily that were induced in P-deficient roots (Table III). Of these, TC 2883 had the highest similarity (93%) to Arabidopsis *PHR1*, a MYB gene implicated in the P deficiency response process. Three Arabidopsis genes have recently been documented to be involved in signal transduction and regulation of P acquisition/homeostasis. These genes encode WRKY75, PHO2 (an E2 conjugase), and SIZ1 (a SUMO E3 ligase; Miura et al., 2005; Aung et al., 2006; Bari et al., 2006; Devaiah et al., 2007). Common bean TCs 2419, 1095, and 2445 have high similarity to WRKY75, PHO2, and SIZ1, respectively. Although none of the bean TCs cited above had enhanced expression in P-stressed roots of common bean, their similarity of Arabidopsis P-signaling genes suggests that comparable bean TCs may play similar roles in bean. Noticeable is that all of the ESTs comprising TC 2419 and 1095 are derived from the P-stressed root library. Interestingly, TC 1622, which is up-regulated

Table V. *Primers and conditions used for semiquantitative RT-PCR*

Target Gene	EST/TC No.	GenBank Accession No. of EST	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Product Size	Annealing Tm/Cycles
					bp	
Peroxidase	TC397	CV542921	CCA ACC AAA CAC TTG CCA ATG	GAG TAG TAG GCC TTG TCG AAT	313	58°C/20
Glycolipid transfer protein-like	TC1903	CV543709	GTT GTT CTC AGT CTG CGA TCA	TAT TGG AGT GGA TGG CAA CGA	751	60°C/25
Translationally controlled tumor protein	TC63	CV542788	CGC TCC GCA CCA GTT ATC A	GGA TCA GTG GCA CCG TCC TTG	528	60°C/25
No BLAST hit	RTS_103_E03	CV541966	GGC TTC AAA ATC CTC ACG C	GCT TGT TGC TAT CTC AAA CGC	214	60°C/20
UDP-Glc-6-dehydrogenase	TC1804	CV541174	GGC TTT ATG TTC TTC TAT GTT	TGA AAC CCT CAA ATA TTA CTC	138	52°C/25
Cytosolic aldehyde dehydrogenase	TC733	CV542619	CCG TGT GCT CGT TCA AG	ATC TCC TCC TGC ACT ATT CTC	282	52°C/25
Senescence-related dihydroorotate dehydrogenase	RTS_109_B06	EH792671	ATA GCA CTT GGG AAG GTG ATG	GTCTTTGTCAGATTG CAAGCC	359	55°C/25
ACC oxidase	TC280	CV542243	TGG CAC CAA AGT TAG CAA CTA	ATT CTG GTG CCA TCC GTT TGA	270	57°C/25
Hypothetical protein	TC1992	CV542243	AAC AGT CAA AAG TAT CAC ACG	GAT TCA GGG TTT CAG TAT AGG	219	55°C/25
Isocitrate dehydrogenase (NADP)	TC1864	CV541923	CCC TGA CGG AAA GAC TAT TGA	ACC TTT ACT TTC TGC GGT GCC	593	60°C/20
S-adenosyl Met decarboxylase	TC83	CV543847	TAC TTG GGA CTG TTG TTA TCA	TAA ACT CAC GGG TAT AGC TTG	1,589	60°C/20
Ribosomal protein 40 S	TC1613	CV543229	TTT CTA GGG TTT CGC TCG GTA	TAA ACA GAA ACA AAC GCC ATA	600	60°C/25
Transporter-like protein	TC2875	CV543019	TGC TTC TTC GCC ACA ACT CAA	CCT TCC AAG ATG TAG CCG TCA	476	60°C/25
Trehalose-6-P synthase	RTS_114_F03	CV542030	TTT CTA AGA ATG AAG CGA GAT	GAT CCA TCA GTT GTT TCG GTA	116	52°C/25
Phosphoribosylanthranilate transferase	RTS_139_D07	CV543777	TGG CCA TGC AGA TTG TG	GGA TTA ACA TTA TGC GAT GAA	144	52°C/25
Caffeine-induced death protein 1	RTS_143_D10	CV544078	AAG CAA AAG CAA TTA GTG GC	ATAGCAAGGCAAACAT CAATG	134	57°C/25
AMP diaminase	TC1176	CV543772	GAC ACT TAT GCC GGT TAT T	GGC GAT CAC ACC ATG CGT TAC	136	52°C/25
Multidrug resistance protein 11	TC2799	EH791098	CAG CAG TGT GAA ATT AAT GGC	GTT TTT GGG GGT GGA TTG CAT	111	57°C/25
Ubiquitin	TC29	CV543388	CCA TAA CTC TTG AGG TGG AGA G	CTT CCC AGT CAA GGT CTT GAC	480	55°C/25

in P-deficient bean roots, encodes a putative UBC-ligase related to a pepper *CaPUB1* that has been implicated in resistance to abiotic stress (Cho et al., 2006).

Studies with white lupin (Uhde-Stone et al., 2003; Liu et al., 2005) and Arabidopsis (Nacry et al., 2005; Karthikeyan et al., 2006; Müller et al., 2007) have shown that sugars and P stress signaling are closely interrelated. Rychter and Randall (1994) found that within 2 weeks of P stress, common bean partitioned more sugars to roots than nonstressed plants. The enhanced expression of P stress-induced genes requires the presence of available sugars. Deprivation of sugars by either shading or stem girdling blocks the expression of P stress-induced genes (Liu et al., 2005). Our metabolic analysis of bean P-stressed roots provides additional support for the role of sugars in P stress. Several sugars (Table IV) were more abundant in P stress roots as compared to P-sufficient roots, suggesting that sugars may be partitioned preferentially to P-stressed roots to support the expression of P stress-induced genes. It is noteworthy that PRL1-associated protein, encoded by CV543658, which has enhanced expression in P stress bean roots, is known to interact with SNF1 to derepress Glc metabolism, stimulate starch accumulation, and inhibit root elongation (Bhalerao et al., 1999). These traits are characteristic of P-stressed plants.

It is also worthwhile to note the reduced amounts of organic acids in P-stressed roots as compared to P-sufficient roots (Table IV). It is well known that P-stressed legume roots release organic acids as a P-adaptive mechanism (Johnson et al., 1996; Neumann and Römheld, 1999; Shen et al., 2002; Dong et al., 2004). Release of organic acids into the rhizosphere enhances P_i solubilization, making P more available. The reduced

amount of organic acids in P-deficient roots more than likely reflects exudation from the root into the rhizosphere. The altered organic acid content of P-stressed roots is also reflected in the reduced expression of TC 1864 isocitrate dehydrogenase-ICD (Table II). This enzyme is a key regulatory enzyme in the tricarboxylic acid cycle. Reduced expression of ICD would lead to a buildup of malate acids that could be available for exudation into the rhizosphere.

Almost 23% of the genes showing enhanced expression in P-stressed bean roots encode proteins having roles in either stress/defense or secondary metabolism (Table I). Hammond et al. (2003) have shown that many genes that respond to P stress in Arabidopsis shoots also respond to other environmental challenges, including salinity, wounding, pathogen attack, anoxia, and other nutrient stresses. In bean, P stress results in the induction of oxidative responses, including increased lipid peroxidation, elevated peroxide levels, and increased catalase and peroxidase activity (Juszczuk et al., 2001). Our results confirm and extend this observation by showing that genes encoding proteins in several aspects of oxidative stress have enhanced expression in P-stressed roots. Moreover, several genes implicated in plant response to diseases, such as PR and NBS-LRR resistance, are up-regulated in bean P-stressed roots along with genes involved in phenylpropanoid synthesis (Table I). Similar patterns of gene activation have been noted for plants undergoing potassium, zinc, iron, and N deficiency stress (Wang et al., 2002; Armengaud et al., 2004; Shin et al., 2005; van de Mortel et al., 2006).

Because enhanced P_i transporter gene expression is highly indicative of the P_i stress response (Raghothama, 1999; Smith, 2001), it was surprising that we did not find any P_i transporter to be highly expressed in P-stressed common bean roots. In fact, we found only a single P_i transporter EST derived from the P-stressed root library. The lack of P_i transporter ESTs in the root library could reflect that the library was made from roots of 21-d-old P-stressed plants. Perhaps earlier sampling dates would have yielded more P_i transporters. On the other hand, we did detect enhanced expression of other types of transporters, including a putative aquaporin, an ATP-binding cassette transporter, and an acetylglucosamine transporter (Table I).

As demonstrated in Figure 1C and previously shown in numerous studies, the root to shoot ratio increased in P-stressed plants as compared to P-sufficient plants. The ratio change was due in part to proliferation of lateral roots in P-stressed plants. Modified root architecture in response to P stress has been noted previously in common bean (Rychter and Randall, 1994; Lynch, 1995; Ge et al., 2000; Liao et al., 2001; Lynch and Brown, 2001) and Arabidopsis (López-Bucio et al., 2003; Ma et al., 2003; Wu et al., 2003). Phosphate starvation was recently shown to induce determinant root development programs in Arabidopsis (Sánchez-Calderón et al., 2005). Recently, quantitative trait loci for root architecture traits that correlate with P acquisition have been identified in bean, strengthening the importance of root structure for low P adaptation (Beebe et al., 2006). Modification of root architecture in response to P deficiency results from the interplay between internal balance of the phytohormones auxin, cytokinin, and ethylene (Gilbert et al., 2000; Williamson et al., 2001; Al-Ghazi et al., 2003; López-Bucio et al., 2003; Ma et al., 2003; Karthikeyan et al., 2006). As one might expect, we found several genes in bean roots related to phytohormone biosynthesis and activity to be responsive to P. Accompanying increased lateral root growth, genes involved in cell wall synthesis and growth were responsive to P.

Reduced shoot growth accompanied by reduced photosynthetic rate (Fig. 1) was symptomatic of P stress in bean. Phosphate content and photosynthesis are related in several ways, and alteration of photosynthesis as a result of P starvation has been shown for several plant species, including common bean (Rychter and Randall, 1994; Mikulska et al., 1998). It has been shown that tobacco plants grown under P deficiency have reduced photosynthate demand in sink organs, resulting in carbohydrate accumulation and decrease in net photosynthesis (Pieters et al., 2001). Our data support the proposition of Morcuende et al. (2007) that repression of photosynthesis may be a secondary response linked to lower demand of photosynthate and higher sugar levels during P limitation.

The results from this work provide an abundance of candidate genes with diverse function that are postulated to play important roles in adaptation of common bean plants to P deficiency. These newly identified genes may be of utility in marker-assisted selection for P-efficient genotypes. The identified candidate genes expand the current information available on the regulation and signaling pathways during P deficiency in plants. In future studies, we propose to define the precise roles of selected candidate genes using reverse genetics approaches.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The common bean (*Phaseolus vulgaris*) Mesoamerican 'Negro Jamapa 81' was used in this study. Plants were grown in controlled-environment (26°C–28°C, 16-h photoperiod) greenhouses at Centro de Ciencias Genómicas/Universidad Nacional Autónoma de México (Cuernavaca, México) and Max Planck Institute of Plant Molecular Physiology (Golm, Germany), or in growth chambers at the University of Minnesota (St. Paul). Surface-sterilized seeds were germinated at 30°C for 3 d over sterile wet filter paper and then planted in pots with vermiculite or coarse quartz sand. Pots were watered 3 d per week with the plant nutrient solution reported by Summerfield et al. (1977). For −P conditions, K_2HPO_4 concentration of the plant nutrient solution was reduced from 1 mM to 5 μM. In −P conditions, cotyledons from each plant were cut 1 week after planting. Plants were grown for 3 weeks before harvesting. Roots for RNA isolation were harvested directly into liquid nitrogen and stored at −80°C.

Soluble P_i Concentration

Soluble P_i content was determined in leaves, stems, and roots from plants grown for 3 weeks in −P or +P conditions using the colorimetric assay

reported by Taussky and Shorr (1953). For each assay, tissues were harvested, weighed, and immediately homogenized in 10 N TCA. For each determination, 12 replicates were analyzed. These were derived from three independent experiments with plants grown in similar conditions with four replicate assays from each treatment (−P roots or +P roots) per experiment.

Photosynthesis and Photosynthetic Pigments Content

The relationship between CO_2 assimilation rate (P_n), increasing C_i, and stomatal conductance and resistance was determined using a portable photosynthesis system (LI-6200 Primer; LI-COR) in −P- versus +P-treated plants. The measurements from mature bean trifolia were undertaken in a greenhouse maintaining leaf temperature and photosynthetically active photon flux density at 25°C and 1,600 μmol m^{-2} s^{-1}, respectively. Each point represents the average of 12 determinations from three independent experiments with plants grown in similar conditions and four replicate assays from each treatment (−P roots or +P roots) per experiment. The CO_2 assimilation rate was adjusted to each leaf area value.

Photosynthetic pigments were extracted from freshly harvested, fully expanded leaves using 80% (v/v) acetone. Carotenes and chlorophyll (*a* and *b*) were determined spectrophotometrically at 470, 663, and 646 nm wavelength, respectively, as reported (Wellburn, 1994).

EST Sequencing and Annotation

Because the macroarrays used in this study were spotted prior to sequencing, 65 of the spotted clones had poor quality sequence and were not included in sequence-based analyses (Ramírez et al., 2005; TIGR/DFCI, Quackenbush et al., 2001) or submitted to GenBank. To include these clones in our analyses, the clones were resequenced. DNA sequencing was performed at the Advanced Genetic Analysis Center (University of Minnesota) and at the Center for Genomic Sciences/Universidad Nacional Autónoma de México (Cuernavaca, México). The new sequences were submitted to GenBank (accession nos. EH791054–EH791109, EH792671–EH792678, and EH795233).

To assign newly sequenced ESTs to existing TCs in the TIGR/DFCI Common Bean Gene Index, the EST sequences were compared to the TCs using TBLASTX (Altschul et al., 1997). To confirm the placement of the EST with the putative matching TIGR/DFCI TC, the overlap of both sequences was checked using the SeqManII program in the DNASTAR software package. Sequence matching indicated that the analyzed bean sequence belonged to the TC, as indicated in Tables I and II. To annotate the sequences described in Tables I to III, all sequences were cross referenced with the TIGR/DFCI Common Bean Gene Index to find the corresponding TCs or singletons. TC or singleton sequences were compared to the Uniprot (version July 2006; Apweiler et al., 2004) protein database using BLASTX and an E-value cutoff of $E < 10^{-4}$.

Nylon Filter Arrays, Hybridization, and Data Analysis

The preparation of a cDNA library from roots from P-deficient bean 'Negro Jamapa 81' plants and the sequence of ESTs (4,329) have been reported (Ramírez et al., 2005; Graham et al., 2006). For macroarray preparation, the cDNA portion of each root EST was amplified by PCR using standard T3 and T7 primers. The PCR products were spotted onto Gene Screen Plus membranes (NEN Life Science Products) using the Q-bot (Genetix) automated spotting system.

Total RNA was isolated from 4 g frozen roots (as reported by Chang et al. [1993]) from plants grown under similar −P or +P conditions in four independent experiments. Radiolabeled cDNA probes were synthesized from total RNA (30 μg) by RT, as reported (Ramírez et al., 2005). Hybridization and washing conditions of nylon filters were performed as reported (Ramírez et al., 2005). Ten independent nylon filter arrays were hybridized with cDNA from each treatment.

Hybridized filters were exposed to phosphor screens for 5 d, and the fluorescent intensity of each spot was quantified as reported (Ramírez et al., 2005; Tesfaye et al., 2006). To work with highly reproducible experiments, linear regression analysis was performed for each pair of membrane replicas; only those replicas for which the linear model could explain at least 80% of the variation ($r^2 \geq 0.8$) were considered. This process yielded a total of six well-correlated replicas for each treatment: −P roots and +P roots, respectively. Array data were normalized and quantified using GeneSpring (version 7.2; Silicon Genetics), as provided by the Supercomputing Institute at the University of Minnesota. *t* tests were performed with a *P*-value cutoff of ≤0.05.

RT-PCR Analysis for Verification of Array Analysis

Total RNA for RT-PCR was isolated from 3 g frozen roots using the RNeasy isolation kit (Qiagen). Quantification of transcripts was performed using two-step RT-PCR following the manufacturer's directions (Ambion and Invitrogen) using poly thymine deoxynucleotide primer. The sequences of oligonucleotide primers and conditions used in RT-PCR reactions are shown in Table V. RT-PCR products were resolved in 1% (w/v) agarose gels in Tris-acetate-EDTA buffer, along with a 1-kb DNA-standard ladder (Invitrogen). Amplification of ubiquitin gene was used as control for uniform PCR conditions.

TF Gene Selection and RT-PCR Primer Design

Genes (EST/TC) coding for proteins specifically involved in transcriptional regulation were selected from the TIGR/DFCI Common Bean Gene Index (www.tigr.org). For protein domain prediction, Inter-Pro Release 11 (www.ebi.ac.uk/interpro) was used. The text of all Inter-Pro database entries was searched for the specific strings "*transcription*", "*DNA*binding*", and "*zinc*finger*" using the SRS search tool (www.ebi.ac.uk/interpro/search.html). The identified domains were assembled in a list. The list was supplemented by Inter-Pro domains that are components of the Gene Ontology (GO) branches "Transcription factor activity" (GO:0003700), "Transcriptional activator activity" (GO:00165643), "Transcriptional repressor activity" (GO:0016564), and "Two-component response regulator activity" (GO:0000156). The GO-Inter-Pro mappings were found using the QuickGO browser on the Inter-Pro page (www.ebi.ac.uk/ego/). In total, 1,533 domains of proteins potentially involved in transcriptional regulation were selected.

Subsequently, all common bean sequences were analyzed for the occurrence of these domains using Inter-ProScan (www.ebi.ac.uk/Inter-ProScan). In 372 sequences, 41 of the preselected domains were found. The Inter-Pro descriptions of these domains were evaluated to select the domains of proteins that are involved in transcriptional regulation.

RT-PCR primers were generated for the 372 TF genes with TIGR's Primer Design Pipeline, which was designed with the aims of high throughput and specificity. The pipeline iterates through three phases: design, specificity, and selection.

First, the design phase queried every region of the target TF sequences with sliding windows to generate primer set candidates that fit the experimental requirements. Each sliding window was 250 bp across and stepped 50 bp along the target sequence per iteration. The experimental requirements were enforced by the following MIT Primer3 (Rozen and Skaletsky, 2000) parameters: PRIMER_MIN_TM 58, PRIMER_OPT_TM 60, PRIMER_MAX_TM 62, PRIMER_SELF_ANY 6, PRIMER_SELF_END 2, PRIMER_MAX_POLY_X 3, and PRIMER_PRODUCT_SIZE_RANGE'60 to 150'.

Next, the specificity phase aligned primer candidates via WU-Blast (W. Gish, 1996–2004; http://blast.wustl.edu) to the TIGR/DFCI Common Bean Gene Index. The selection phase then discarded primer candidates that registered possible secondary hits, defined as specificity alignments that achieved 80% or greater identity over the length of the primer and included at least one of the terminal ends of the primer in the alignment. The remaining, qualifying primer sets were further prioritized by self-complementarity and poly-X characteristics to achieve selection of the most preferred primers for every target.

The primer design pipeline was implemented in object-oriented Perl modules and supported by a relational MySQL database. Sequences of primer pairs used to amplify each TF gene are shown as supplemental data.

Real-Time RT-PCR Conditions and Analysis of Bean TF

Total RNA for real-time RT-PCR was isolated from 400 mg frozen roots based on the protocol reported by Heim et al. (1993). Three biological replicas were isolated for each treatment (−P and +P roots), extracting RNA from different sets of plants grown in similar conditions. RNA concentration was measured in NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and 10 μg of total RNA was digested with TURBO DNase (catalog no. 1907, Ambion), adding ribonuclease inhibitor (catalog no. N211B, Promega) and following the manufacturer's directions. Absence of genomic DNA contamination was subsequently confirmed by real-time PCR amplification, using primers designed for the bean E2 *UBC9* reference gene (TC362; primers: F, 5'-GCTCTCCATTTGCTCCCTGTT-3'; R, 5'-TGAGCAATTTCAGGCACCAA-3'). cDNA was synthesized using SuperScriptIII reverse transcriptase (Invitrogen), according to manufacturer's instructions. The efficiency of cDNA

synthesis was assessed by real-time PCR amplification of control *UBC9* gene. Only cDNA preparations that yielded similar C_T values (i.e. 19 ± 1) for the reference gene were used for comparing TF transcript levels.

Quantitative determinations of relative transcript levels of TF genes using RT-PCR were carried out at the Max Planck Institute of Molecular Plant Physiology (Golm, Germany) according to Czechowski et al. (2004). Each real-time RT-PCR run for the whole set of TF genes (372) plus reference (housekeeping) and marker genes was performed in a 384-well plate. The bean phosphatase gene (TC201), which is highly induced in P-deficient roots (Ramírez et al., 2005), was included as a P deficiency marker in every real-time PCR run. The primers used for phosphatase gene amplification are: F, 5′-GCCCAAGTTT-GAGGCTGAAAG-3′; R, 5′-TCAAGTCCCACACCGGAAAGT-3′. TF gene expression was normalized to that of UBC9, which was the most constant of the four housekeeping genes included in each PCR run. −P/+P average expression ratios were obtained from the equation $(1 + E)_T^{\Delta\Delta C}$, where $\Delta\Delta C_T$ represents $\Delta C_{T(-P)} - \Delta C_{T(+P)}$, and E is the PCR efficiency. Student's *t* test was performed with a *P*-value cutoff at 0.05.

Plant Metabolite Extraction

Plant metabolite extraction from root samples of −P- and +P-treated bean plants and GC-MS metabolite profiling was done as reported previously for *Lotus japonicus* (Colebatch et al., 2004; Desbrosses et al., 2005). Twelve replicate samples each of roots from plants grown under +P and −P conditions were harvested from pods, rinsed with tap water, dried on filter paper, and shock frozen in liquid nitrogen. Frozen samples of 60 to 150 mg fresh weight (FW) were ground 3 min in 2-mL micro vials with a clean stainless steel metal ball (5-mm diameter) using a Retschball mill set at 20 cycles s^{-1}. Grinding components of the mill were cooled with liquid nitrogen to keep samples deep frozen. Frozen powder was extracted with hot MeOH/$CHCl_3$ and the fraction of polar metabolites prepared by liquid partitioning into water and further processed as described (Desbrosses et al., 2005).

GC-Time of Flight-MS Metabolite Profiling

GC-time of flight (TOF)-MS profiling was performed using a FactorFour VF-5ms capillary column, 30 m long, 0.25 mm i.d., 0.25 μm film thickness with a 10-m EZ-guard precolumn (Varian BV), and an Agilent 6890N gas chromatograph with splitless injection and electronic pressure control (Agilent) mounted to a Pegasus III TOF mass spectrometer (LECO Instrumente). Details of GC-TOF-MS adaptation of the original profiling method (Desbrosses et al., 2005) are described by Wagner et al. (2003) and Erban et al. (2006). Metabolites were quantified after mass spectral deconvolution (ChromaTOF software version 1.00, Pegasus driver 1.61; LECO) of at least three mass fragments for each analyte. Peak height representing arbitrary mass spectral ion currents of each mass fragment was normalized using the amount of the sample FW and ribitol for internal standardization of volume variations to obtain normalized responses (per gram FW) and response ratios as described (Colebatch et al., 2004; Desbrosses et al., 2005).

Identification of Metabolites within GC-MS Metabolite Profiles

Metabolites were identified using the NIST05 mass spectral search and comparison software (National Institute of Standards and Technology; http://www.nist.gov/srd/mslist.htm) and the mass spectral and RI collection (Schauer et al., 2005) of the Golm Metabolome Database (Kopka et al., 2005). Mass spectral matching was manually supervised, and matches were accepted with thresholds of match >650 (with maximum match equal to 1,000) and RI deviation <1.0%. Information on the polar metabolites, using the corresponding mass spectral identifiers can be found at http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_smq.html. Metabolites are characterized by chemical abstracts system identifiers and compound codes issued by the Kyoto Encyclopedia of Genes and Genomes (Kanehisa et al., 2004). Metabolites were identified by standard substances or by MSTs. The term MST is used for repeatedly occurring yet nonidentified compounds, which can be recognized by mass spectrum and RI, as defined earlier (Colebatch et al., 2004; Desbrosses et al., 2005). MSTs are characterized and named by best mass spectral match to compounds identified by NIST05 or Golm Metabolome Database using match value and hit name (Table IV). The response ratio −P to +P for each metabolite/MST was calculated dividing the average metabolite concentration from 12 samples from roots of P-deficient plants over the average metabolite concentration from 12 samples from roots of control plants (Table IV).

ICA and Statistical Analysis

ICA (Scholz et al., 2004) was applied to metabolite profiles (as compiled in supplemental data). Data were normalized by calculation of response ratios using the median of each metabolite as denominator and subsequently subjected to logarithmic transformation. Missing value substitution was as described earlier (Scholz et al., 2005). Statistical testing was performed using the Student's *t* test. Logarithmic transformation of response ratios was applied for approximation of required Gaussian normal distribution of metabolite profiling data.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EH791054 to EH791109, EH792671 to EH792678, and EH795233.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Root transcript levels of all the genes in the common bean macroarray.

Supplemental Table S2. Complete list of common bean TF gene and primer sequences.

Supplemental Table S3. Root transcript levels of all common bean TF genes determined by real-time RT-PCR.

Supplemental Table S4. Complete metabolic profile response from common bean roots.

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Global Changes in the Transcript and Metabolic Profiles during Symbiotic Nitrogen Fixation in Phosphorus-Stressed Common Bean Plants[1][W][OA]

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Phosphorus (P) deficiency is widespread in regions where the common bean (*Phaseolus vulgaris*), the most important legume for human consumption, is produced, and it is perhaps the factor that most limits nitrogen fixation. Global gene expression and metabolome approaches were used to investigate the responses of nodules from common bean plants inoculated with *Rhizobium tropici* CIAT899 grown under P-deficient and P-sufficient conditions. P-deficient inoculated plants showed drastic reduction in nodulation and nitrogenase activity as determined by acetylene reduction assay. Nodule transcript profiling was performed through hybridization of nylon filter arrays spotted with cDNAs, approximately 4,000 unigene set, from the nodule and P-deficient root library. A total of 459 genes, representing different biological processes according to updated annotation using the UniProt Knowledgebase database, showed significant differential expression in response to P: 59% of these were induced in P-deficient nodules. The expression platform for transcription factor genes based n quantitative reverse transcriptase-polymerase chain reaction revealed that 37 transcription factor genes were differentially expressed in P-deficient nodules and only one gene was repressed. Data from nontargeted metabolic profiles indicated that amino acids and other nitrogen metabolites were decreased, while organic and polyhydroxy acids were accumulated, in P-deficient nodules. Bioinformatics analyses using MapMan and PathExpress software tools, customized to common bean, were utilized for the analysis of global changes in gene expression that affected overall metabolism. Glycolysis and glycerolipid metabolism, and starch and Suc metabolism, were identified among the pathways significantly induced or repressed in P-deficient nodules, respectively.

A key to the success of the legume family, which comprises approximately 700 genera with more than 18,000 species (Doyle and Luckow, 2003), was the evolution of mutualistic symbioses with nitrogen (N)-fixing bacteria of the family Rhizobiaceae to directly capture atmospheric dinitrogen (N_2) to support plant growth. Symbiotic nitrogen fixation (SNF) takes place in specialized rhizobia-induced legume root nodules and involves a tight association between the two symbionts. SNF and legume crop production might be affected by disease and insect pressures but also by edaphic constraints that include climatic conditions, nutrient deficiency, soil acidity, and metal toxicity.

Phosphorus (P) is an essential macronutrient for plant growth and development, with P concentration ranging from 0.05% to 0.5% plant dry weight. P is taken by the plants as phosphate (Pi), but Pi is unevenly distributed and relatively immobile in soils. As

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a result, crop yield in 30% to 40% of arable land is limited by P availability (Vance et al., 2003). Widespread P deficiency is a major restriction for SNF and legume crop productivity (Andrew, 1978).

N_2-fixing legumes require more P than legumes growing on mineral N, but little is known about the basis for the higher P requirement. Growing root nodules are strong P sinks in legumes. For example, P concentration in the nodules of soybean (*Glycine max*; Sa and Israel, 1991) and white lupin (*Lupinus albus*; Schulze et al., 2006) from P-deficient plants can reach up to 3-fold that of other plant organs. The deleterious effect of P deficiency on SNF and plant growth has been evidenced through the evaluation of physiological and biochemical parameters, such as nodule number and mass, nitrogenase and carbon/N assimilatory enzyme activities, CO_2 fixation, photosynthesis, energy status, organic acid synthesis, release of protons or organic acids, and nodule O_2 diffusion. Such studies have been performed in different legume species such as lupin, soybean, alfalfa (*Medicago sativa*), white clover (*Trifolium repens*), *Medicago truncatula*, pea (*Pisum sativum*), and common bean (*Phaseolus vulgaris*; Jakobsen, 1985; Israel, 1987; Sa and Israel, 1991; Ribet and Drevon, 1995a, 1995b; Al-Niemi et al., 1997; Vadez et al., 1997; Almeida et al., 2000; Tang et al., 2001, 2004; Hogh-Jensen et al., 2002; Olivera et al., 2004; Schulze and Drevon, 2005; Schulze et al., 2006; Le Roux et al., 2008).

Common bean is the world's most important grain legume for direct human consumption. P deficiency is widespread in the bean-producing regions of the Third World and is perhaps the factor that most limits N_2 fixation on small farms. Bean genotypes differ in N_2 fixation ability and P use efficiency under P deficiency. Considering the greater P need of nodulated legumes, P-tolerant cultivars that in addition partition a significant percentage of their P uptake to nodules will be a prerequisite for improved bean N_2 fixation (Graham, 1981; Broughton et al., 2003; Graham et al., 2003; Tang et al., 2004).

Plant response to P deficiency and stress tolerance involves multiple genes and intricate regulatory mechanisms. In the case of common bean, two reports discuss gene expression analyses in the roots of P-deficient plants. Hernández et al. (2007) identified 126 P-responsive genes, through transcript profile analysis, and Tian et al. (2007) identified 240 P stress-induced genes, through the analysis of a suppressive subtractive cDNA library. In addition, using our platform for transcription factor (TF) profiling, based on quantitative reverse transcription (qRT)-PCR technology, we identified 17 TF genes that were differentially expressed in P-deficient roots of the common bean (Hernández et al., 2007). We demonstrated that the [AQ4] PvPHR1 TF, which is induced in P-deficient bean roots, and the PvmiR399 microRNA both play essential roles in the P-starvation signaling of the common bean (Valdés-López et al., 2008).

Microarray and macroarray approaches enabled the identification of a large number of genes that are differentially expressed in legume nodules of *M. truncatula*, *Lotus japonicus*, soybean, and bean (Colebatch et al., 2002, 2004; El Yahyaoui et al., 2004; Kouchi et al., 2004; Lee et al., 2004; Asamizu et al., 2005; Ramírez et al., 2005; Starker et al., 2006; Brechenmacher et al., 2008). However, to our knowledge, there are no reports on the transcriptome of P-deficient legume nodules.

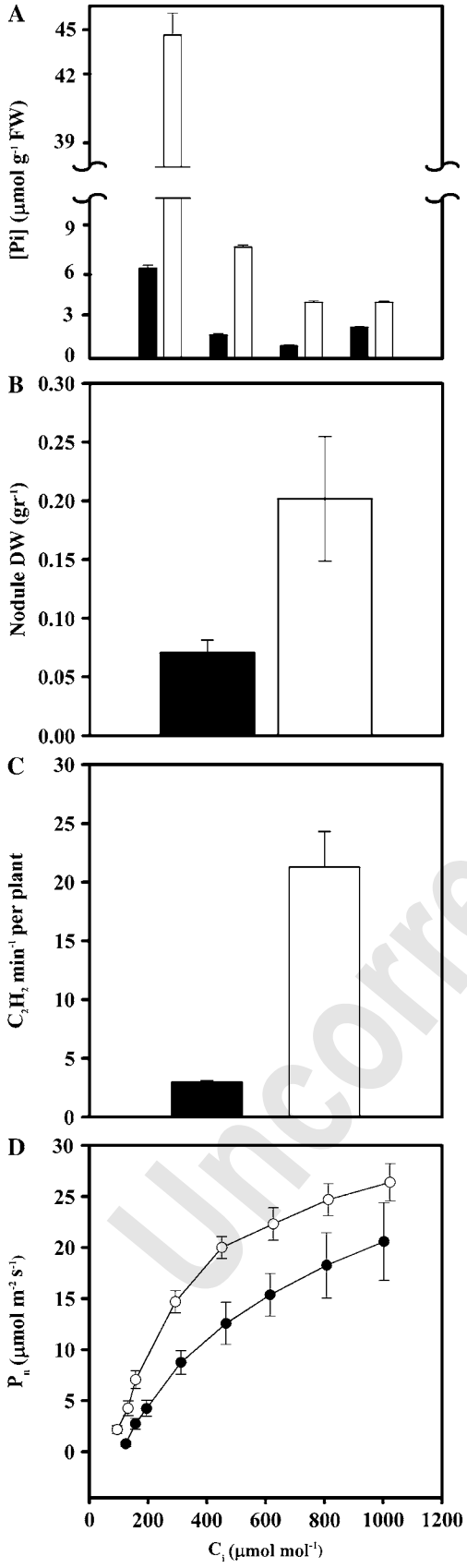
The understanding of the mechanisms for the adaptation to P deficiency of common bean plants under SNF conditions will become useful for future crop improvement. In an attempt to contribute to such efforts, we performed research focused on the identification of genes, gene networks, and signaling pathways that are relevant for P-deficient bean nodules. We undertook a macroarray-based transcript profiling screen of P-deficient bean nodules elicited by *Rhizobium tropici*. Furthermore, we used qRT-PCR to assess nodule gene expression of the whole set of proposed bean TFs (Hernández et al., 2007) in order to identify TFs that may regulate gene expression in P-stressed nodules. Third, we performed a nontargeted metabolite profiling of bean nodules using gas chromatography-mass spectrometry (GC-MS) and correlated metabolic changes to the orchestrated global changes of gene transcription as a response to P starvation.

In order to interpret the gene expression data, we used the MapMan (Thimm et al., 2004) and PathExpress (Goffard and Weiller, 2007b; Goffard et al., 2009) bioinformatics tools, which were adapted to the common bean. PathExpress allowed identification of the differentially expressed genes that were assigned EC numbers and thus associated to the relevant metabolic pathways operating in P-deficient bean nodules. Such metabolic pathways inferred by transcriptome analysis were additionally associated to some of the discovered P deficiency-responsive metabolites. The overall goal of our research was to identify candidate genes that may be useful to bean improvement and that will contribute to the understanding of the acclimation to P deficiency of the N_2-fixing common bean.

RESULTS

Phenotypic Characterization

Germinated common bean seedlings were inoculated with *R. tropici* CIAT899 and then subjected to long-term P deficiency (–P) under an otherwise controlled environment using a 200-fold lower Pi concentration as compared with P-sufficient (+P) control plants. The performance of the plants was assessed 21 d post inoculation (dpi) and exposure to the –P condition. Control plants accumulated higher concentrations of soluble Pi in leaves (7-fold), stems (4-fold), and roots (4-fold) but only 1.5-fold in nodules as compared with –P plants (Fig. 1A). As expected,



nodulation and SNF were affected in –P bean plants. These plants showed 3.5-fold less nodule mass (Fig. 1B) and 85% reduction in nitrogenase-specific activity (Fig. 1C).

The content of photosynthetic pigments such as chlorophyll *a* and *b* and carotenes was similar in plants under –P and +P treatments (data not shown). However, P-deficient plants exhibited an inhibition of the net photosynthetic rate (P_n). P_n was 40% lower at ambient CO_2 concentrations (350 μmol mL^{-1}) and reflected the lower carboxylation efficiency under –P conditions (Fig. 1D). The maximum P_n was not significantly affected in –P plants, indicating that the Rubisco and ribulose 1,5-bisphosphate regeneration was maintained. The latter observation suggests that symbiotic P-deficient bean plants were capable of regulating photosynthetic activity.

Macroarray Analysis for Nodule Response to P Deficiency

Global gene expression in P-deficient bean nodules as compared with control P-sufficient nodules was determined by macroarray analyses. Two different macroarrays were prepared by spotting nylon filters with ESTs from the common bean –P root and mature nodule cDNA libraries (Ramírez et al., 2005; Graham et al., 2006). The root and nodule macroarrays included 2,212 and 1,786 unigene sets, respectively, as reported (Ramírez et al., 2005; Hernández et al., 2007).

Total RNA was isolated from plants grown under similar conditions as described for each treatment (−P or +P). Eight nylon filter root arrays and eight nodule arrays were hybridized with radiolabeled first-strand cDNA synthesized from four independent sources of total RNA. From the eight hybridizations, four replicates of each array and of each treatment, with high determination coefficients ($r^2 \geq 0.8$), were chosen for the analysis of differential gene expression. A total of 459 genes (tentative consensus sequences [TCs] or singletons) showed significant ($P \leq 0.05$) differential expression in P-deficient nodules (Supplemental Tables S1 and S2).

In order to aid gene annotation, cDNAs were assigned to TCs (Dana Farber Cancer Institute [DFCI] *Phaseolus vulgaris* Gene Index [PhvGI], version 2.0). The annotation of all ESTs from the nodule and root cDNA library ESTs was updated by comparison (BLASTX; E value $< 10^{-4}$) with the UniProt Knowl-

Figure 1. Effect of P deficiency on bean in symbiosis with *R. tropici*. A, Soluble Pi in different plant organs. FW, Fresh weight. B, Nodule dry weight (DW). C, Nitrogenase activity determined by acetylene reduction assay. D, Net photosynthesis (P_n) rate as a function of changing internal CO_2 (C_i). Plants inoculated with *R. tropici* CIAT899 were grown for 21 d under P deficiency (black bars or circles) or P-sufficient conditions (white bars or circles). Values are means ± SE of 12 determinations from three independent experiments with four replicates per experiment.

edgebase (UniProtKB) database (release 14.1; UniProt Consortium, 2008; Supplemental Table S3). From the 7,129 total EST sequences, 5,102 ESTs had significant best matches to UniProtKB/Swiss-Prot, 621 ESTs had significant best matches to UniProtKB/trEMBL but not to Swiss-Prot, while 1,406 ESTs did not have significant matches to UniProtKB. A UniProt keyword was assigned to each EST. The biological process was the preferred keyword; ESTs were classified in 39 different biological processes (Supplemental Table S4). If this keyword was not available, other keywords such as the molecular function or the cellular component were assigned (Supplemental Table S4).

Supplemental Tables S1 and S2 show the genes that were induced (263) or repressed (196) 2-fold or more in P-deficient nodules. These genes were initially grouped in four main categories: metabolism, cell cycle and development, interaction with the environment, and unknown function. The latter includes genes with similarity to a hypothetical protein or DNA sequences with unknown function and those for which no BLAST hit was found. Figure 2 shows the more relevant biological processes that group the genes differentially expressed in P-deficient nodules.

The induced genes (Supplemental Table S1) were classified into the categories metabolism (30%), cell cycle and development (6%), interaction with the environment (34%), and unknown function (30%). The biological processes statistically overrepresented in the set of induced ESTs, compared with the remaining ESTs, were Arg metabolism, autophagy, auxin signaling pathway, and plant defense (Fig. 2; Supplemental Table S1). Several biological processes from the carbon (one-carbon metabolism, glycolysis, gluconeogenesis, pentose shunt, gluconate utilization), N (Arg and purine metabolism), and lipid (lipid synthesis, lipid metabolism, lipid degradation) metabolisms showed high proportions of induced ESTs (Fig. 2).

The most abundant category among the repressed genes (Supplemental Table S2) was interaction with the environment (41%), followed by metabolism (25%), unknown function (24%), and cell cycle and development (10%). "Nucleotide metabolism" was the only biological process that was statistically overrepresented in the set of repressed ESTs (Fig. 2; Supplemental Table S2). In contrast to the main induced biological processes, several processes from N metabolism (nucleotide metabolism and biosynthesis, protein biosynthesis) showed a high proportion of repressed ESTs, similar to processes like cell cycle and cell wall biosynthesis and degradation (Fig. 2).

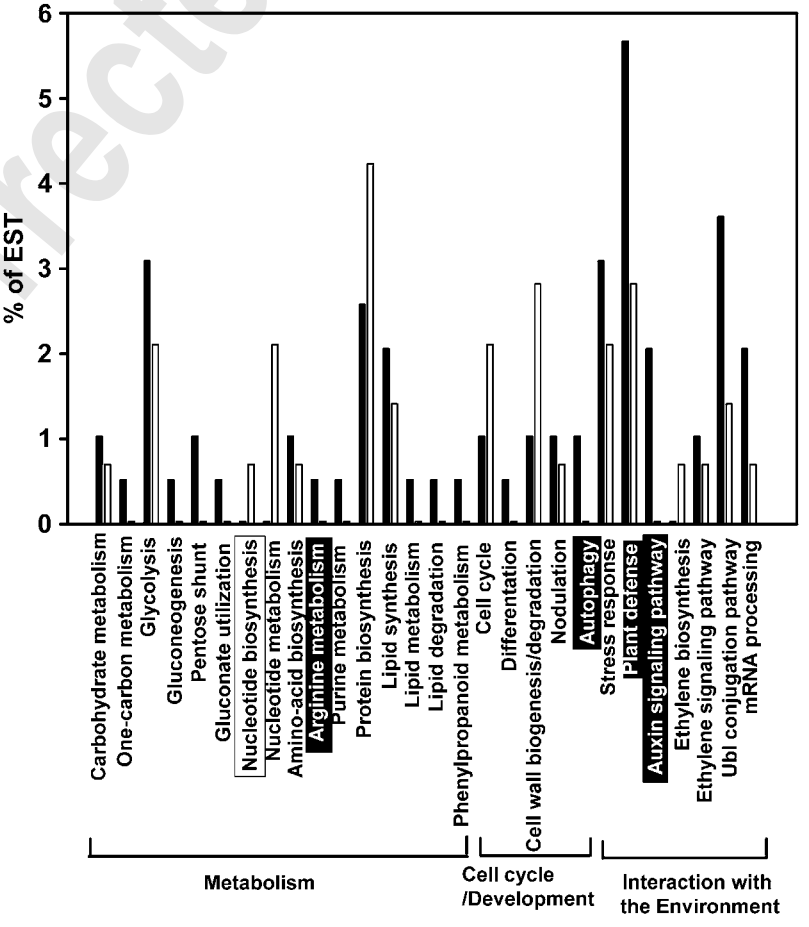


Figure 2. Distribution of selected bean ESTs into biological processes according to UniProtKB keywords (UniProt Consortium, 2008). Black bars, ESTs induced in –P nodules; white bars, ESTs repressed in –P nodules. The percentage represents the proportion of submitted ESTs that have been assigned in the corresponding category. The biological processes overrepresented in the set of induced or repressed ESTs, compared with the remaining ESTs (Supplemental Table S4), are highlighted with black or white boxes, respectively. Main functional categories that group the different biological processes are indicated.

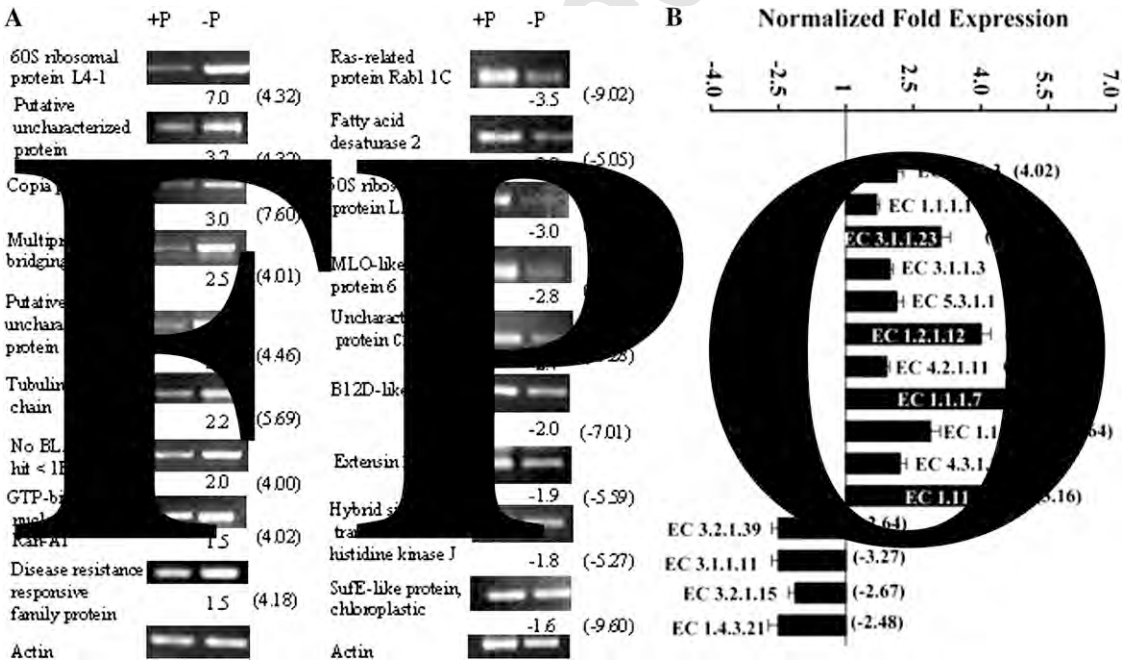
Expression Analyses of Selected Genes by RT-PCR

Nine –P nodule-induced ESTs and nine repressed ESTs were randomly selected from Supplemental Tables S1 and S2 in order to confirm the macroarray expression data by semiquantitative RT-PCR (sRT-PCR). The selected genes corresponded to different functional categories and biological processes and showed high –P/+P expression ratios in macroarray analysis (≥ 4 or ≤ -4). In addition, 15 genes assigned as enzymes that participate in significantly induced or repressed metabolic pathways (see below) were selected to confirm their macroarray expression data by real-time qRT-PCR. These included the gene (NOD_210_H10) annotated as malate dehydrogenase (EC 1.1.1.7), which was not detected as significantly induced through macroarray analyses but participated in the glycolysis/gluconeogenesis significantly induced pathway (see below). Primers and conditions for RT-PCR analyses are given in Supplemental Table S5.

As shown in Figure 3, all of the genes that were tested for expression responses using sRT-PCR or qRT-PCR gave results that confirmed the expression results obtained with the macroarray experiment regarding the induction or repression of each gene in P-deficient nodules. However, there was a variation of –P/+P expression ratios for each tested gene when comparing the values obtained from macroarray with those from RT-PCR; in general, the values obtained from macroarray analysis were higher (Fig. 3). The latter may be related to the different sensitivity of the technologies used.

TF Transcript Profiling by qRT-PCR

We identified a set of 372 TF genes from common bean that were selected from DFCI PhvGI (version 1.0) and had been included into the reported qRT-PCR platform of TF expression profiling (Hernández et al., 2007). We used this platform to determine the differential expression of bean TF genes that might be involved in regulating the gene expression response of P-deficient nodules. Three biological replicates of –P- and +P-treated nodules were analyzed. In each qRT-PCR run, the phosphatase gene (TC3168), known to be induced in –P bean roots (Ramírez et al., 2005; Hernández et al., 2007), was included as a P-deficiency control of the –P response in bean nodules. This gene was highly induced in nodules and had an average



[AQ15] **Figure 3.** Verification of macroarray results by sRT-PCR and qRT-PCR analyses. A, Selected genes identified as induced (left panel) or repressed (right panel) in P-deficient nodules were evaluated by sRT-PCR. The actin gene was included as a control for uniform RT-PCR conditions (bottom). The intensity of the bands was quantified densitometrically, and the –P/+P normalized expression ratios are shown below each gel image. B, Selected genes assigned to represent enzymes (with indicated EC numbers) induced or repressed in P-deficient nodules. Enzyme names corresponding to each EC number are indicated in Supplemental Tables S1 and S2 and Figures 6 and 7. Values represent the normalized –P/+P fold expression as the average of three biological replicates ± SD. For ratios lower than 1 (genes repressed in P deficiency), the inverse of the ratio was estimated and the sign was changed. The –P/+P expression ratios obtained from the macroarray analyses (Supplemental Tables S1 and S2) are shown in parentheses. The primer sequences and reaction conditions for sRT-PCR and qRT-PCR analyses are presented in Supplemental Table S5.

expression ratio comparing –P with +P of 30.12 (P = 1E-6), as determined by qRT-PCR. Thus, the P-deficient status of the nodules under investigation was confirmed. Table I shows the 37 TF genes that were differentially expressed, 2-fold or more ($P \leq 0.05$), in P-deficient nodules. These genes were classified into 17 different TF families according to the Arabidopsis (*Arabidopsis thaliana*) TF classification (Riechmann, 2002). Only one TF gene from the C3H-type 1(Zn) family was repressed; all others were induced. None of

Table I. *TF genes significantly expressed in nodules of P-deficient plants identified by real-time RT-PCR*

GenBank Accession No. (TC No.)	Annotation	BLASTX E Value	TF Family/Domain	Expression Ratio –P/+P	*P*
Induced in –P					
CV531158	O23379 Putative zinc finger protein CONSTANS-LIKE 11	1.00 E-8	C2C2(Zn)	5.55	0.00130
CB542189	Q96502 Zinc finger protein CONSTANS-LIKE 2	1.00 E-8	C2C2(Zn)	2.71	0.04174
TC3558	Q96288 Salt tolerance protein	1.00 E-76	C2C2(Zn)	2.33	0.00672
CB540841	Q9SSE5 Zinc finger protein CONSTANS-LIKE 9	3.00 E-7	C2C2(Zn)	2.23	0.01626
BQ481766 (TC4839)[a]	Q96502 Zinc finger protein CONSTANS-LIKE 2	1.00 E-9	C2C2(Zn)	2.00	0.00360
TC3525	Q42430 Zinc finger protein 1 (WZF1)	2.00 E-13	C2H2(Zn)	3.37	0.00122
CB541538	B9IEY1 Predicted protein	6.00 E-40	C2H2(Zn)	3.31	0.01795
TC4594	Q42430 Zinc finger protein 1 (WZF1)	4.00 E-149	C2H2(Zn)	2.57	0.03902
CV542423	Q39266 Zinc finger protein 7	4.00 E-07	C2H2(Zn)	2.25	0.01362
TC6164	Q8L6Y4 Polycomb group protein EMBRYONIC FLOWER 2	1.00 E-149	C2H2(Zn)	2.06	0.01031
CV533267	Q9FJ93 Dehydration-responsive element-binding protein 1D	2.00 E-41	AP2/EREBP	4.70	0.01197
BQ481785	Q84QC2 Ethylene-responsive transcription factor ERF017	3.00 E-34	AP2/EREBP	2.71	0.05405
CB540147 (TC6825)[a]	Q8LC30 Ethylene-responsive transcription factor RAP2-1	2.00 E-39	AP2/EREBP	2.55	0.05779
TC6676	O80337 Ethylene-responsive transcription factor 1A	3.00 E-63	AP2/EREBP	2.15	0.02622
TC3112	Q9SAH7 Probable WRKY transcription factor 40	4.00 E-74	WRKY(Zn)	2.76	0.01113
CX129652	Q8S8P5 Probable WRKY transcription factor 33	3.00 E-52	WRKY(Zn)	2.50	0.03801
TC3738	Q9SUP6 Probable WRKY transcription factor 53	3.00 E-57	WRKY(Zn)	2.02	0.04625
TC4946	Q9LX82 Transcription factor MYB48	6.00 E-25	MYB superfamily	2.36	0.01668
TC2999	Q6ZNW5 UPF0580 protein C15orf58	6.00 E-40	MYB superfamily	2.32	0.01079
DN153793	Q9M2Y9 Transcription factor RAX3	1.00 E-20	MYB superfamily	2.04	0.05997
CV536452	Q9FIW5 Putative NAC domain-containing protein 94	8.00 E-27	NAC	2.34	0.03376
CV534675	Q5CD17 NAC domain-containing protein 77	3.00 E-30	NAC	2.26	0.03562
CV530141	B9RH27 Transcription factor, putative	4.00 E-11	NAC	2.04	0.02866
TC7761	Q9LMA8 Protein TIFY 10A	1.00 E-30	ZIM/TIFY	3.40	0.01489
TC5396	Q9LMA8 Protein TIFY 10A	1.00 E-39	ZIM/TIFY	2.48	0.00604
TC7287	Q9FXG8 BEL1-like homeodomain protein 10	9.00 E-48	Homeobox	2.69	0.04828
TC3707	Q6YWR4 Homeobox-Leu zipper protein HOX16	4.00 E-24	Homeobox	2.18	0.00083
CV538920	P24068 Ocs element-binding factor 1	7.00 E-21	bZIP	2.27	0.00289
TC3839	Q69IL4 Transcription factor RF2a	3.00 E-39	bZIP	2.21	0.02188
BQ481439	Q0PJI4 MYB transcription factor MYB138	1.00 E-52	Homeodomain-like	6.83	0.04473
CV536165	Q9SMX9 Squamosa promoter-binding-like protein 1	1.00 E-54	SBP	4.38	0.00422
TC6556	P13089 Auxin-induced protein AUX28	2.00 E-99	AUX/IAA	3.37	0.00049
TC6822	Q8VY21 Tubby-like F-box protein 3	1.00 E-153	TUB	2.86	0.03833
TC5176	Q69VG1 Chitin-inducible gibberellin-responsive protein 1	1.00 E-153	GRAS	2.58	0.02628
CV529418	O49403 Heat stress transcription factor A-4a	2.00 E-27	HSF	2.13	0.01078
CV529563 (TC3533)[a]	Q9CAA4 Transcription factor BIM2	8.00 E-50	bHLH	2.00	0.03141
Repressed in –P					
CV530991	Q0D3J9 C3H53_ORYSJ Zinc finger CCCH domain-containing protein 53	2.00 E-12	C3H-type 1(Zn)	−2.56	0.00581

[a]Previous singletons (in PhvGI version 1.0) now correspond to the indicated TC according to PhvGI version 2.0.

the differentially expressed TF genes detected by qRT-PCR analysis was detected by macroarray analysis, indicating the much higher sensitivity and accuracy of the qRT-PCR platform for TF expression profiles (Table I; Supplemental Tables S1 and S2). Most of the induced TF genes (10) belong to the C2C2(Zn) or the C2H2(Zn) family, and three belong to the MYB superfamily. The participation of MYB TFs in P-starvation signaling is known for Arabidopsis (Rubio et al., 2001; Todd et al., 2004). We have demonstrated earlier the respective role of a MYB TF in bean roots: PvPHR1 was reported to be relevant for the P-starvation signaling by Valdés-López et al. (2008). A TF from the ZIM/TIFY family, TC7761, was the only TF gene found to be induced both in nodules (Table I) and in roots (Hernández et al., 2007) of P-deficient bean plants.

Metabolome Analyses

Nontargeted metabolite profiling of bean roots using GC-MS was performed in order to assess the degree to which changes in plant gene expression in P-deficient bean nodules affect metabolism. The complete information of 81 covered mostly primary metabolites and nonidentified mass spectral metabolite tags (MSTs) detected in bean nodules when subjected to –P and +P treatments is provided as Supplemental Table S6. Thirty-nine of the identified metabolites and MSTs showed a response ratio higher than 1, indicating an increase in P-deficient nodules, while 31 showed a decrease in –P nodules. Eleven of the detected metabolites and MSTs were not affected by the nutrient stress (response ratio = 1; Supplemental Table S6).

Table II shows those metabolites and MSTs (45) included in significantly induced or repressed pathways (see below), those with –P/+P response ratios higher than 1.5-fold, and those with lower but significant ($P \leq 0.05$) ratios. In agreement with previous analyses (Desbrosses et al., 2005; Hernández et al., 2007), the identified metabolites were mostly primary metabolites belonging to the following compound classes: amino acids, N compounds, organic acids, polyhydroxy acids, sugar phosphates, polyols, and sugars. Most of the carbon metabolites, such as organic and polyhydroxy acids, sugars, and polyols were increased significantly in P-stressed nodules, while most of the amino acids and other N compounds showed a decrease in P-stressed nodules. As expected, phosphates such as Fru-6-P, Glc-6-P, and glycerate-6-phosphate were also decreased in P-starved nodules (Table II).

The quantitative data on the relative pool size changes of the metabolites listed in Supplemental Table S6 were subjected to independent component analysis (ICA). A major difference of the metabolic phenotype between P-deficient and P-sufficient nodules was revealed using an ICA scores plot (Fig. 4). This analysis of the metabolite response ratios of all observed metabolites in 12 samples from P-deficient nodules and 12 samples of P-sufficient nodules allowed unambiguous partitioning into two sample groups, showing the clear metabolic differentiation of –P-stressed individual plants from the P-sufficient metabolite phenotype (Fig. 4).

Transcriptome and Metabolome Data Analyses

The data of differentially expressed genes from P-stressed nodules, generated in this work through macroarray analyses and TF gene profiling, were analyzed using the MapMan (Thimm et al., 2004) and PathExpress (Goffard and Weiller, 2007b; Goffard et al., 2009) software tools, which allow visualization and interpretation of the data in the context of known biological networks. For this task, both software tools were customized to the common bean as described (see "Materials and Methods").

For MapMan data analyses, a recently created soybean mapping (S. Yang, unpublished data) was the basis for a common bean mapping file containing the differentially expressed genes resulting from the current macroarray and TF profiling approaches (Supplemental Table S7). After submitting the –P/+P expression ratios of the determined bean genes, different graphical representations were obtained for visual analysis from MapMan. To avoid an overlap with the PathExpress investigation, the MapMan analysis focused on the maps describing pathways other than the metabolic. Figure 5 shows the bean nodule MapMan graph representation of the regulation overview map. As was expected from our manual gene expression results, the majority of the genes assigned to the different categories in the regulation overview map were induced. Evident abundant categories, which included most of the induced regulatory genes, were TFs, receptor kinases, and protein degradation. In addition, several genes from the overrepresented induced biological processes, auxin signaling pathway and autophagy (Fig. 2), are included in the regulatory categories from Figure 5.

The input files for the PathExpress analysis comprised the list of genes that were differentially expressed in P-deficient bean nodules (Supplemental Tables S1 and S2). PathExpress uses the subset of submitted genes that can be assigned EC numbers and reports all metabolic networks that include these EC numbers as well as the enzymes in these networks that correspond to submitted identifiers. Table III shows the list of significant ($P < 0.05$) pathways or subpathways that were induced or repressed in P-stressed bean nodules. The enzymes assigned to the significantly induced or repressed pathways from Table III are highlighted in Supplemental Tables S1 and S2, respectively. Since PathExpress graphical representations of metabolic pathways contain two types of nodes, enzymes labeled with EC numbers and metabolites labeled with Kyoto Encyclopedia of Genes and Genomes (KEGG) identifiers (Kanehisa et al., 2004), we analyzed the graph representations to link in-

Table II. *Selected metabolites identified by GC-MS in bean nodules from –P- and +P-treated plants*

Metabolite	RI, Expected	RI, SD	Response Ratio −P/+P[a]
Amino acids			
Gly	1,304.5	−0.17	**−1.4**
β-Ala	1,424.7	−0.12	3.5
Ser	1,252.9	0.09	**−1.2**
Asn	1,665.8	−0.02	**2.0**
Thr	1,290.9	−0.02	**−2.0**
4-Hyp	1,518.0	0.21	**−1.4**
Gln	1,767.6	−0.05	**−1.5**
Leu	1,151.0	0.52	**1.6**
Lys	1,847.3	−0.02	1.2
Phe	1,553.1	0.15	**2.4**
N compounds			
Putrescine (agmatine)[b]	1,737.2	−0.24	**−1.4**
Pipecolic acid	1,366.6	−0.16	**−1.6**
Picolinic acid	1,327.2	0.20	**1.5**
Spermidine	2,251.1	−0.22	**−1.6**
Urea	1,235.6	0.06	**−1.4**
Organic acids			
Malonic acid	1,195.0	0.30	**1.4**
Tartaric acid	1,625.9	0.03	**−1.7**
Malic acid	1,477.3	0.11	1.5
2,4-Dihydroxybutanoic acid	1,403.6	−0.08	−2.1
cis-Aconitic acid	1,740.5	−0.02	**1.6**
Citric acid	1,804.6	−0.04	**1.2**
Vanillic acid	1,761.6	0.10	**1.7**
Polyhydroxy acids			
Glyceric acid	1,321.7	−0.02	−1.1
Threonic acid	1,546.4	−0.04	**1.4**
Galactonic acid-1,4-lactone	1,877.4	−0.31	**1.3**
Galactaric acid	2,032.5	−0.22	**1.5**
Galactonic acid	1,984.7	−0.29	**1.8**
Gulonic acid	1,951.4	−0.21	**2.0**
Phosphates			
Glyceric acid-3-phosphate	1,790.2	0.06	−1.1
Fru-6-P	2,292.8	−0.03	**−2.2**
Glu-6-P	2,329.6	−0.13	−1.3
Polyols			
Threitol	1,485.1	−0.12	**3.3**
Sugars			
Fru	1,856.2	−0.24	−1.1
Man	1,869.0	−0.17	**1.4**
Suc	2,629.6	−0.28	**2.2**
α,α′-Trehalose	2,730.1	−0.26	**1.6**
MSTs[c,d]			
[516; 1H-Indole-2,3-dione, 1-(tert-butyldimethylsilyl)-5-isopropyl-, 3-(*O*-methyloxime)]	1,691.2	−0.04	**−2.4**
[771; α-D-Methylfructofuranoside (4TMS)]	1,760.9	−0.02	**1.5**
[802; Methylcitric acid (4TMS)]	1,909.57	−0.06	**1.2**
[965; Gluconic acid, 2,3,4,5,6-pentakis-*O*-(trimethylsilyl)-trimethylsilyl ester]	2,002.9	−0.27	**1.6**
[834; 2-*O*-Glycerol-β-D-galactopyranoside (6TMS)]	2,160.3	−0.04	−1.5
[926; Galactosylglycerol (6TMS)]	2,297.2	−0.12	**2.4**
[802; Gulose (5TMS)]	2,424.3	0.26	−1.9
[533; Lactose methoxyamine (8TMS)]	2,878.2	−0.10	**1.3**
[882; Melibiose (8TMS)]	3,092.9	−0.27	**2.6**

[a]The response ratio of average –P nodule response compared with average +P nodule response is listed (*t* test significance of $P < 0.05$ is indicated by boldface numbers for the response ratio). For ratios lower than 1, the inverse of the ratio was estimated and the sign was changed. [b]Represents the sum of two or more metabolites. [c]Reference substance not yet available. [d]MSTs are characterized by match factor and mass spectral hit.

[AQ18]

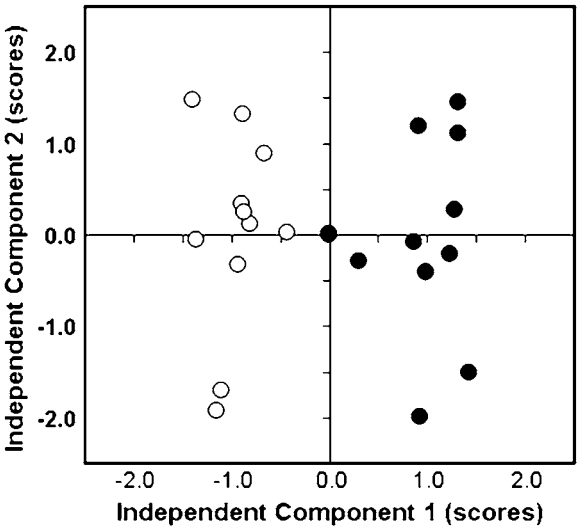


Figure 4. ICA of the major metabolic variances in bean nodules determined by GC-MS-based metabolite profiling. Bean plants grown in P-sufficient (white circles) and P-deficient (black circles) conditions were used. Score analysis demonstrated the clear difference between the P-sufficient and P-deficient metabolic phenotypes.

creased or decreased metabolites, as shown in Table II, to the pathways revealed by gene expression analysis (Table III). Thus, we integrated both transcriptomic and metabolomic data with the known map of metabolic networks. However, such integration was limited to 13 metabolites detected in our analysis that were included in significant metabolic pathways (Tables II and III), while the rest of the detected metabolites (68) belong to other compound classes not included in these pathways (Supplemental Table S6). Figures 6 and 7 show graphical representations of selected induced and repressed pathways, respectively. These visualizations are based on the PathExpress output and include the significant P-responsive enzymes (Fig. 3; Supplemental Tables S1 and S2) and the respective metabolites (Table II), highlighted according to up-regulation (green) or down-regulation (red) or pool concentration. In addition, Figures 6 and 7 include the EC numbers of those enzymes from each pathway that [AQ5] are included in the PvGI and other metabolites from the pathways, albeit undetected in our analysis.

The significantly induced pathway of glycerolipid metabolism is depicted in Figure 6A. This pathway includes four induced enzymes, slightly decreased glycerate, and increased galactosyl-glycerol content in P-deficient nodules. The gene products take part in the biosynthesis of galactolipids such as digalactosyl-diacylglycerol, which has been reported as an important component of plasma membranes from P-deficient plants (Andersson et al., 2003; Tjellström et al., 2008). A common plant response to P starvation is the modification of membrane lipid composition by increasing polar lipid production with low Pi content, such as galacto- and sulfo-lipids (Essigmann et al., 1998; Hartel et al., 2000; Andersson et al., 2003, 2005; Tjellström et al., 2008).

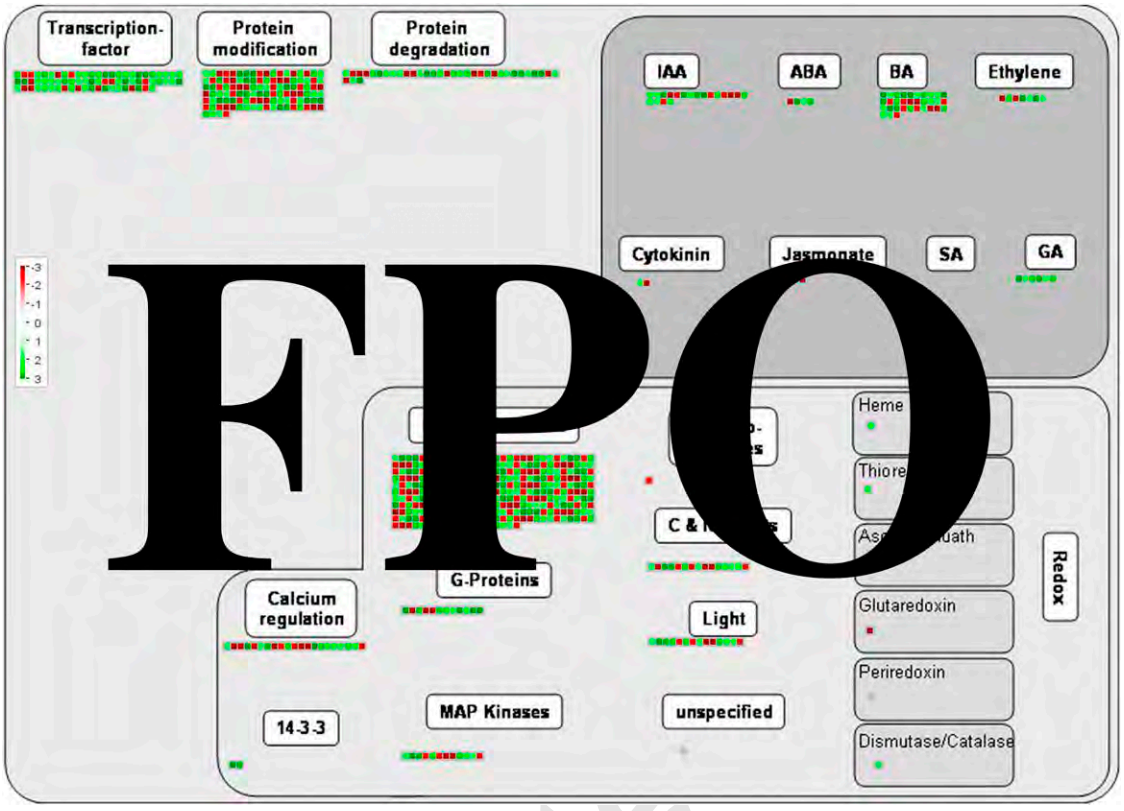
Symbiotic carbon supply is a key plant process of nodule metabolism that is facilitated mainly by a high production of organic acids that are offered to the bacteroid symbiont for enabling efficient N_2 fixation. Figure 6B depicts the induced glycolysis/gluconeogenesis/carbon fixation pathway, which includes six induced enzymes, slightly decreased Glc-6-P, decreased Fru-6-P, and slightly increased malate contents in P-stressed nodules. This pathway is in agreement with what has been demonstrated for malate synthesis in legume nodules, involving mainly CO_2 fixation through phospho*enol*pyruvate carboxylase and malate dehydrogenase, rather than through the tricarboxylic acid cycle (Vance and Heichel, 1991). [AQ6]

Although the content of several amino acids was reduced in –P nodules, Phe was increased more than 2-fold (Table II) and the metabolic pathway for this amino acid was accordingly induced (Table III). Figure 6C shows the details of the Phe pathway with three –P-induced enzymes.

Figure 7 depicts two significantly repressed metabolic pathways. The starch and Suc pathway includes four down-regulated enzymes, indicating the repression of starch and pectin biosynthesis and a rechanneling of carbon toward synthesis of soluble sugars, such as the increasing Suc and α,α-trehalose pools, as well as increased gulonate in P-stressed nodules (Fig. [AQ7] 7A). The subpathway of β-Ala metabolism, depicted in Figure 7B, was significantly repressed in –P nodules. The repression of two enzymes from this pathway correlates with the increase in β-Ala and the decreased spermidine content observed (Table II). From the significantly repressed Lys biosynthesis pathway (Table III), Lys was found increased (Table II) and the enzyme diaminopimelate decarboxylase, which converts this amino acid to meso-2,6-diaminoheptane dioate, was down-regulated (Supplemental Table S2).

DISCUSSION

A low P level in the soil is an important constraint for bean production, especially in Latin America and Africa (Graham, 1981). In order to understand the molecular responses of bean for adaptation to P deficiency, we have analyzed the root transcriptomic and metabolomic profiles of P-stressed bean plants (Hernández et al., 2007). Considering that P deficiency is one of the most limiting factors for SNF (Andrew, 1978; Graham, 1981; Graham et al., 2003), in this work we undertook functional genomic approaches to advance the understanding of the adaptation of *R. tropici*-inoculated bean plants to P stress. Transcript and metabolic responses were analyzed from mature bean nodules of P-deficient plants with evident deleterious effects on nodulation and SNF (Fig. 1).



[AQ16] **Figure 5.** MapMan regulation overview map showing differences in transcript levels between P-deficient and P-sufficient bean nodules. In the color scale, green represents higher gene expression and red represents lower gene expression in P-deficient [AQ17] nodules as compared with control (+P) nodules. The lists of normalized expression values are given in Table I and Supplemental Tables S1 and S2. The complete sets of genes submitted to MapMan analysis are given in Supplemental Table S7.

The P-deficient inoculated bean plants analyzed showed much lower soluble Pi concentration in different plant organs as compared with control (P-sufficient) plant organs (Fig. 1). However, Pi was higher in nodules than in stems or roots of P-stressed bean plants (Fig. 1). This observation is in agreement with previous reports indicating that, particularly under P deficiency, nodules are strong sinks for P and show higher P concentration in nodules than other organs (Sa and Israel, 1991; Al-Niemi et al., 1997; Vadez et al., 1997; Tang et al., 2001; Hogh-Jensen et al., 2002; Schulze et al., 2006). It has been reported that N_2 fixation tolerance to P deficiency varies among different common bean genotypes (Vadez et al., 1997; Tang et al., 2001). The common bean cv Negro Jamapa 81 used in this work showed a dramatic decrease in nodule mass and in N_2 fixation capacity, as determined by acetylene reduction assay nitrogenase activity per plant (Fig. 1). The latter is in agreement with numerous studies that have reported the negative effects of P starvation on N_2-fixing capacity of legumes (Jakobsen, 1985; Israel, 1987; Sa and Israel, 1991; Al-Niemi et al., 1997; Vadez et al., 1997; Tang et al., 2001, 2004; Olivera et al., 2004; Le Roux et al., 2008). Israel (1987) has postulated that P has specific roles in nodule initiation, growth, and functioning in addition to its involvement in host plant growth processes.

Transcript expression patterns revealed by hybridization of nylon filter arrays spotted with ESTs from bean –P roots and mature nodule cDNA libraries (approximately 4,000 unigene set) resulted in 459 differentially expressed genes with 2-fold or more induction (59% genes) or repression (41% genes) in –P nodules (Supplemental Tables S1 and S2). Most of the significantly up-regulated genes derived from the P-stressed root cDNA library, while the significantly down-regulated genes derived from both libraries (Supplemental Tables S1 and S2). This may be related with a probable biased overrepresentation of genes expressed in this nutrient deficiency. However, RT-PCR of selected induced and repressed genes confirmed their differential expression (Fig. 3). Furthermore, several of the induced genes revealed by macroarray analysis (Supplemental Table S1) have been predicted by Graham et al. (2006) as bean candidate P stress-induced genes through clustering analysis across four legume species and Arabidopsis. The bioinformatically predicted (Graham et al., 2006) and experimentally up-regulated genes detected in this work include glyceraldehyde-3-phosphate dehydro-

Table III. *Significant pathways or subpathways identified by PathExpress analysis*

Pathways that are significantly associated with the list of submitted sequence identifiers with a *P* value threshold of 0.05.

Pathway/Subpathway	No. of Enzymes Included in Bean EST Sequences	No. of Differentially Expressed Enzymes	*P*
Induced			
Glycerolipid metabolism	9	5	0.00222
Fatty acid metabolism	2	2	0.01580
Glycolysis-gluconeogenesis	19	7	0.00496
Carbon fixation	4	4	0.00023
Isoflavonoid biosynthesis	2	2	0.01580
3-Chloroacrylic acid degradation	2	2	0.01580
Metabolism of xenobiotics by cytochrome P450	2	2	0.01580
Phe metabolism	6	3	0.02900
Selenoamino acid metabolism	6	3	0.02900
Bile acid biosynthesis	3	2	0.04350
Drug metabolism, cytochrome P450	3	2	0.04350
Phenylpropanoid biosynthesis	7	3	0.04630
Repressed			
Starch and Suc metabolism	22	4	0.00730
Pentose and glucuronate interconversions	7	2	0.00730
β-Ala metabolism	7	3	0.03920
Aminoacyl-tRNA biosynthesis	9	3	0.03460
Lys biosynthesis	4	2	0.03920

genase, alcohol dehydrogenase, oxidoreductases, wound-induced and pathogenesis-related proteins, Ser/Thr kinases, peroxidases, and MYB and WRKY transcription factors.

The transcript profile of P-deficient noncolonized bean roots revealed 126 differentially expressed genes (Hernández et al., 2007). A comparative analysis between the P-responsive genes from roots (Hernández et al., 2007) and from *Rhizobium*-elicited nodules (Supplemental Tables S1 and S2) showed that only 24 genes, out of 585, have a common response in the two organs. Supplemental Figure S1 shows the results of such comparative analysis, including a flower diagram and the list of common responsive genes. Twelve genes are up-regulated in roots and nodules, and only two genes are down-regulated in both organs (Supplemental Fig. S1). The rest (10 genes) are differentially regulated in roots as compared with nodules (Supplemental Fig. S1). The main functional categories of the genes up-regulated in both –P bean roots and nodules include proteins from regulation/signal transduction processes (i.e. steroid-binding protein, translationally controlled tumor protein, RNA-binding protein), a pathogenesis-related protein, and proteins related to carbon/sugar metabolism or sensing (i.e. monosaccharide-sensing protein, aldehyde dehydrogenase; Supplemental Fig. S1). Specifically, we found that the gene coding for *S*-adenosylmethionine synthase 2 (TC2965) is up-regulated, while the gene coding for *S*-adenosylmethionine decarboxylase proenzyme (TC7398) is down-regulated, in both roots and nodules (Supplemental Fig. S1), pointing to a relevant role of *S*-adenosylmethionine or polyamine metabolism in P-deficiency response of common bean. The very small proportion of common P-responsive genes in bean roots and nodules suggests a rather different response of each organ to the same nutrient stress. From our comparative analysis of transcript and metabolite profiles, we can conclude that the main response of P-deficient roots is addressed to maintain P homeostasis and root architecture modification, while responses of P-stressed nodules are mainly oriented to maintain adequate carbon/N flux between the symbionts and to avoid oxidative stress.

Nontargeted metabolite analysis, based on GC-MS technology, led to the identification of 81 metabolites and MSTs from bean nodules (Supplemental Table S6). Some of the detected metabolites were increased in –P nodules, some were decreased, and some metabolite pools did not change in sufficient versus deficient conditions (response ratio –P/+P = 1; Supplemental Table S6). ICA analysis from the identified metabolites indicated major differences among phenotypes of P-deficient and P-sufficient nodules (Fig. 4). The PathExpress software tool (Goffard and Weiller, 2007b; Goffard et al., 2009) was used in an attempt to provide comprehensive and integrative analyses of the transcript and metabolic responses found in P-stressed bean nodules. We identified relevant metabolic pathways associated both with enzymes coded by a subset of induced or repressed nodule genes and with responsive nodule metabolites (Fig. 3; Tables II and III).

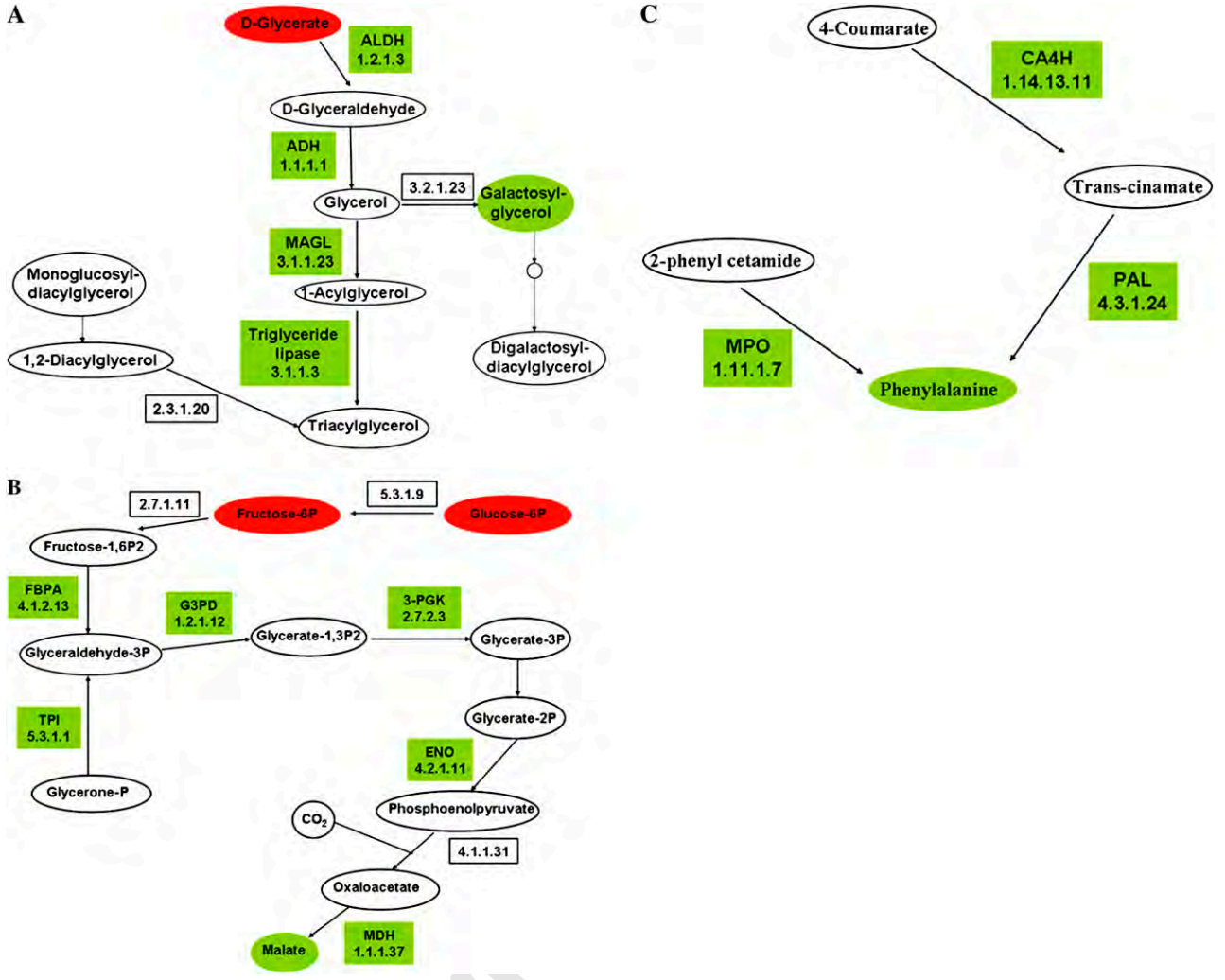


Figure 6. Representation of selected metabolic pathways induced in –P-stressed nodules. Drawings are based on PathExpress outputs (Goffard and Weiller, 2007b; Goffard et al., 2009) and contain two types of nodes: metabolites represented by ellipses, and enzymes (boxes) labeled with the enzyme name or abbreviation and/or the EC number. Enzymes coded by genes included in the PhvGI are shown. The color code indicates the induced gene expression of enzymes (Fig. 3; Supplemental Table S1) or increased metabolite pools (Table II; green) or respective decrease (red). Other metabolites shown in each pathway (white ellipses) were not detected in our analysis. A, Glycerolipid metabolism. ADH, Alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; MAGL, monoglyceride lipase. B, Glycolysis-gluconeogenesis-carbon fixation. ENO, Enolase 2; FBPA, Fru-bisphosphate aldolase; G3PD, glyceraldehyde-3-phosphate dehydrogenase; MDH, malate dehydrogenase; 3-PGK, phospho-glycerate kinase; TPI, triosephosphate isomerase. C, Phe metabolism. CA4H, Trans-cinnamate 4-monooxygenase; MPO, myeloperoxidase; PAL, Phe ammonia lyase.

From the detected metabolites, 13 responsive metabolites could be associated with significantly induced or repressed pathways (Figs. 6 and 7); the rest of the metabolites from these pathways were not detected in our analysis.

Our integrated analyses indicated that the reduction of SNF in P-stressed bean plants led to a reduction of general N metabolism. A decreased –P/+P response ratio was observed in several N metabolites, including the N compounds spermidine, putrescine, and urea, and most of the detected amino acids (Table II). The latter correlates with the diminished expression of three aminoacyl-tRNA enzymes and significant repression of this biosynthesis pathway (Table III; Supplemental Table S2). In addition, the nucleotide metabolism was overrepresented among the repressed biological processes of –P nodules (Fig. 2). These findings contrast with the metabolic response of P-stressed bean noncolonized roots, where a significant increase of amino acid concentration was reported (Hernández et al., 2007). Morcuende et al. (2007) also reported a higher amino acid concentration for P-deprived Arabidopsis seedling cultures, as compared with full-nutrition control cultures. Such a contrasting

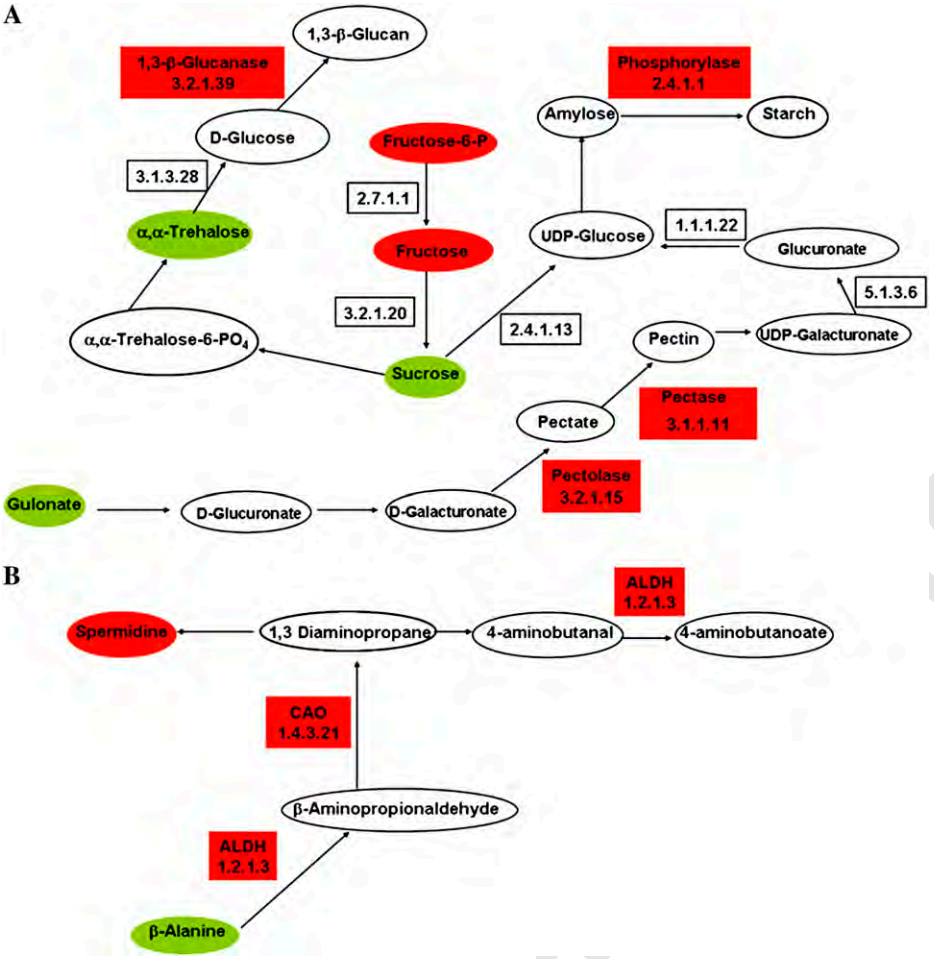


Figure 7. Representation of selected metabolic pathways repressed in –P-stressed nodules (Fig. 3; Tables II and III; Supplemental Table S2). For color-coded representation of the relative changes of metabolite pools and enzyme gene expression, compare with Figure 6. A, Starch and Suc metabolism. B, β-Ala metabolism. ALDH, Aldehyde dehydrogenase; CAO, primary amine oxidase.

response supports the observations of particularly high sensitivity of inoculated legumes, depending solely on fixed N_2, to environmental limitations such as P starvation, which result in diminished nodulation and SNF, as compared with nonsymbiotic plants, which may have N sufficiency in P-deficient soils.

P deficiency in plants alter carbon metabolism in [AQ8] shoot; higher levels of carbon are allocated to the root and thereby increase the root-shoot biomass ratio and alter the root morphology. Some P-starved plant species accumulate sugars in the root and reduce photosynthesis, because sugars exert metabolite feedback regulation, allowing changes in gene expression and excreting organic acids to the rhizosphere as responses for adaptation to stress (for review, see Vance et al., 2003; Hermans et al., 2006). Roots from P-deficient common bean plants showed a decreased concentration of organic acids, which was interpreted as resulting from their exudation to the rhizosphere. The same roots showed accumulation of sugars. This enhanced carbohydrate allocation can be interpreted as the root demand of photosynthate under conditions of decreased net photosynthesis (Hernández et al., 2007). Legume nodules are strong carbon sinks; photosynthate is highly required for symbiotic N_2 fixation and for assimilation of fixed N into amino acids. Carbon and N metabolisms and their interaction/regulation are key processes of nodule function, and the regulation of the gene expression in response to the C:N status has been widely investigated for several years. Data from this work show that P-stressed bean nodules fixed N_2, albeit at a reduced level (Fig. 1). Therefore, we assume that photosynthate is still demanded by –P bean nodules, resulting in low sugar accumulation and high organic acid accumulation (Table II) and maintenance of net photosynthesis (Fig. 1). This –P nodule phenotype contrasts with the reported phenotype of –P bean roots (Hernández et al., 2007). Only Suc, Man, and α,α-trehalose showed a modest increase in the –P/+P response ratio in bean nodules (Table II), while we found six sugars increased in bean roots (Hernández et al., 2007) and several sugars were found accumulated in Arabidopsis under P stress (Misson et al., 2005; Morcuende et al., 2007; Müller et al., 2007). The accumulation of α,α-trehalose in bean nodules might also be related to its role as an osmoprotector or to its function as a signal molecule activating stress tolerance pathways in plants (Paul, 2007). Although some P-stressed plant species accumulate starch in roots (Hermans et al., 2006; Morcuende et al., 2007), we

observed that several enzymes from the starch and Suc metabolic pathways were repressed in –P bean nodules and did not detect accumulation of starch or other carbon polymers (Figs. 3 and 7), suggesting that in stressed nodules, sugars are channeled into glycolysis and organic acid synthesis rather than toward carbon polymer synthesis.

Photosynthate provided to nodules as Suc is metabolized to supply respiratory substrates, mainly malate, to the bacteroids and to provide carbon skeletons for the incorporation of fixed N to amino acids (Vance and Heichel, 1991). Our data show that the glycolysis/carbon-fixation pathway is significantly induced in P-stressed nodules (Fig. 6). This pathway includes the sequential action of phospho*enol*pyruvate carboxylase and malate dehydrogenase, resulting in malate synthesis; this and other organic acids showed an increased –P/+P response ratio (Table II). The point of divergence of glycolysis at phospho*enol*pyruvate serves to circumvent the conventional adenylate-requiring pyruvate kinase and has been interpreted as an adaptive response to P stress for roots and nodules of several plant species (Olivera et al., 2004; Misson et al., 2005; Hermans et al., 2006; Morcuende et al., 2007; Müller et al., 2007; Le Roux et al., 2008). A potential drawback of this branch point would be the competition of organic acids for the tricarboxylic acid cycle and for amino acid synthesis; accordingly, we observed low amino acid concentrations in –P nodules (Table II). Le Roux et al. (2008) reported that excessive malate accumulation in P-deficient lupin nodules may inhibit N_2 fixation and N assimilation, an interpretation that might also hold true for bean P-deficient nodules.

Under P deficiency conditions, plants can remobilize P from internal resources, such as nucleic acids and phospholipids. In this regard, the induction of genes involved in the membrane-phospholipid degradation has been reported in different plant species (Hartel et al., 2000). Some of these genes participate in the galacto- and sulfo-lipid synthesis, which, under P deficiency, are the principal membrane components (Andersson et al., 2003; Tjellström et al., 2008). In Arabidopsis, lipid composition is more sensitive to P deficiency in leaves than in roots (Misson et al., 2005; Morcuende et al., 2007). We detected that monoglyceride lipase and triglyceride lipase genes, involved in galactolipid synthesis, were induced in Pi-deficient root nodules (Figs. 3 and 6; Supplemental Table S1). Although we do not have information about nodule lipid composition under –P conditions, there is evidence indicating that diacylglyceryl, *N*,*N*,*N*-trimethylhomoserine is the principal component of the bacteroid membranes from bean nodules under P deficiency (C. Sohlenkamp, personal communication).

Plant responses to abiotic stress are regulated at different levels, transcriptional and posttranscriptional, with both routes involving intricate signaling pathways. Our bioinformatic analysis based on the MapMan software tool (Thimm et al., 2004) revealed several cellular signaling and regulatory processes that involve a number of bean nodule P-deficient response genes (Fig. 5). Most of these genes, induced in –P nodules, included receptor and mitogen-activated protein kinases, genes involved in protein modification/degradation, in calcium regulation, and in phytohormone regulation, as well as TF genes (Fig. 5). Similar types of regulatory genes are induced in P-stressed Arabidopsis (Wu et al., 2003; Misson et al., 2005; Morcuende et al., 2007; Müller et al., 2007). [**AQ9**]

In this work, we found that 37 of the 372 identified bean TF genes (Hernández et al., 2007) were differentially expressed, and only one was repressed, in P-deficient nodules, as revealed by the TF expression platform based on qRT-PCR (Table I). TFs are master regulators of gene expression. In P deficiency, around 100 TFs were differentially expressed in Arabidopsis plants, while in bean roots, only 17 of 372 analyzed TFs showed differential expression (Wu et al., 2003; Misson et al., 2005; Hernández et al., 2007). The P-deficient root-responsive TFs belong to different gene families, comprising *MYB*, *SCARECROW*, *AP2*, *F-box*, *HOMEOBOX*, *WRKY*, *NAC*, *ERF/AP2*, *NAM*, and *C2H2 Zinc-finger* (Wu et al., 2003; Misson et al., 2005; Hernández et al., 2007; Müller et al., 2007). Some of the bean nodule P-responsive TFs have been implicated in specific responses to P deficiency in other plant species (i.e. members of the WRKY and C2H2 ZFP TF families) that are involved in the root architecture modification and in the regulation of some –P-responsive genes (Devaiah et al., 2007a, 2007b). Also, several TFs that respond in –P conditions in different species and organs, including bean roots and nodules, are additionally implicated in other stresses, such as drought (*NAC*, *AP2/EREBP*), pathogenesis (*WRKY*, *TIFY*), and salinity (*C2C2 ZFP*). These data revealed the cross talk of different signaling pathways in the adaptation to P deficiency.

Our data showed the induction of members of the AP2/EREBP and TIFY TF families in P-stressed bean nodules (Table I). The role of these TFs in legumes might be related to root and nodule developmental processes, since AP2/EREBP and TIFY TFs have been implicated in ethylene and jasmonic acid phytohormone signaling pathways, respectively (Kizis et al., 2001; Chini et al., 2007; Thines et al., 2007), and AP2/EREBP has been implicated in the root and nodule development in *L. japonicus* (Asamizu et al., 2008). There are no reports on the involvement of TIFY TFs in P-deficiency plant response. We are using reverse genetic approaches to investigate the function of a TIFY TF in the response of bean roots and nodules to P deficiency; preliminary results show that modification of *TIFY* gene expression affects the nodulation of bean plants (G. Hernández, unpublished data).

This work presents integrative analyses of transcript and metabolic expression data from stressed bean nodules in an attempt to provide important insight into the P-starvation response. However, the integration of transcriptomics with metabolomics, proteo-

mics, and enzyme biochemistry will be needed to achieve a thorough understanding of the intricate mechanisms by which plant metabolism adapts to nutritional P deficiency. Our results provide an abundance of candidate regulatory genes and candidate metabolic pathways that are postulated to play important roles in the adaptation of symbiotic bean plants to P deficiency and that may be used for marker-assisted selection of P-efficient bean genotypes. To make relevant contributions to develop better N_2-fixing bean genotypes, it is imperative to consider the improvement in both N use and P use. Information generated here combined with future studies, including direct and reverse genetic analyses, might lead to the long elusive goal of improving N_2 fixation in agronomically important grain legumes.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The common bean (*Phaseolus vulgaris*) Mesoamerican cv Negro Jamapa 81 was used in this study. Plants were grown during spring in controlled-environment greenhouses (26°C–28°C, 16-h photoperiod) at the Centro de Ciencias Genómicas/Universidad Nacional Autónoma de México (Cuernavaca, México) and the Max Planck Institute of Plant Molecular Physiology (Golm, Germany) or in growth chambers at the University of Minnesota (St. Paul). Surface-sterilized seeds were germinated at 25°C over sterile, wet filter paper. Three days postimbibition, seeds were sown in pots with vermiculite or coarse quartz sand and inoculated with *Rhizobium tropici* CIAT899 as reported (Ramírez et al., 2005). Pots were watered 3 d per week with Summerfield plant nutrient solution without N (Summerfield et al., 1977). For –P conditions, K_2HPO_4 concentration of the plant nutrient solution was reduced from 1 mM to 5 μM. In –P conditions, cotyledons from each plant were cut 5 d after planting. Plants were grown for 21 dpi before harvesting. Nodules for RNA isolation were harvested directly into liquid N and stored at –80°C.

Soluble Pi Concentration, Nitrogenase Activity, and Photosynthesis

Soluble Pi content was determined at 21 dpi in different organs of plants grown in –P or +P conditions as reported (Taussky and Shorr, 1953; Hernández et al., 2007). Nitrogenase activity was determined in detached, 21-dpi nodulated roots by the acetylene reduction assay. The relationships between CO_2 assimilation rate (net photosynthetic rate) and increasing internal CO_2, stomatal conductance, and resistance were determined using a portable photosynthesis system (LI-6200 Primer; LI-COR) in –P- versus +P-treated plants as reported (Hernández et al., 2007). Each value represents the average of 12 determinations from three independent experiments with plants grown in similar conditions and with four replicate assays from each treatment (–P or +P) per experiment.

EST Sequencing and Annotation

Because the macroarrays used in this study were spotted prior to sequencing, 82 of the spotted clones had poor-quality sequence and were not included in sequence-based analyses (Ramírez et al., 2005; DFCI PhvGI) or submitted to GenBank. In order to include these clones in our analyses, the clones were resequenced. DNA sequencing was performed at the Advanced Genetic Analysis Center (University of Minnesota). The new sequences were submitted to GenBank (accession nos. GO355314–GO355395).

The annotation of all EST sequences from the nodule and P-deficient root common bean cDNA libraries (DFCI PhvGI), including the newly sequenced ESTs (7,129 sequences), was updated by comparing with proteins from the UniProtKB database (http://www.uniprot.org, release 14.1; UniProt Consortium, 2008) using BLASTX. The best match, with a threshold *E* value of 1.00E-4, was selected and UniProtKB keywords were extracted; both were assigned to each EST (Supplemental Table S3). The sequences described in Supplemental Tables S1 and S2 were cross-referenced with the DFCI PhvGI (version 2.0) to find the corresponding TCs or singletons.

Nylon Filter Arrays and Hybridization

The preparation of cDNA libraries from P-deficient roots and from mature nodules from Negro Jamapa 81 bean plants and the sequences of ESTs have been reported (Ramírez et al., 2005; Graham et al., 2006). Two different macroarrays, with the ESTs from each library (root macroarray and nodule macroarray), were prepared as reported (Ramírez et al., 2005; Hernández et al., 2007).

Total RNA was isolated from 0.5 g of mature (21-dpi) nodules from inoculated bean plants grown under similar –P or +P conditions in four independent experiments. Synthesis of radiolabeled cDNA probes from 30 mg of total RNA and hybridization and washing conditions of nylon filters were as reported (Ramírez et al., 2005). Eight independent nylon filter root macroarrays and eight independent nodule macroarrays were hybridized with cDNA from each treatment: –P nodules and +P nodules.

Hybridized filters were exposed to phosphor screens for 5 d for root macroarray and for 2 d for nodule macroarray, and the fluorescent intensity of each spot was quantified as reported (Ramírez et al., 2005). The signal intensity of each spot was determined automatically using the software ArrayPro Analyzer (Media Cybernetics). To work with highly reproducible experiments, linear regression analysis was performed for each pair of membrane replicas; only those replicas for which the linear model could explain at least 80% of the variation ($r^2 \geq 0.8$) were considered. This process yielded four well-correlated replicas for each macroarray (root or nodule macroarray) and for each treatment. The housekeeping genes ubiquitin-conjugating enzyme (TC8137) and ubiquitin (TC5422) served as internal normalization controls for calculating expression ratios between the treatments from the root macroarray and the nodule macroarray, respectively. Each TC included more than one EST spotted in each array; the chosen housekeeping genes showed constant intensity values for all of the ESTs from each TC. The average intensity value from the ESTs of each TC was used for normalization for each macroarray. Student's *t* test for paired observations was applied to determine whether genes showed significant differential expression values (*P* value cutoff at 0.05) from each treatment. sRT-PCR and qRT-PCR approaches were used to verify macroarray expression data. Total RNA for RT-PCR was isolated from 0.5 g of frozen 21-dpi nodules. Quantification of transcripts by sRT-PCR was performed using two-step RT-PCR following the manufacturer's directions (Ambion) using a polythymine deoxynucleotide (dT) primer. Amplified sRT-PCR products were resolved on 2% (w/v) agarose gels in Tris-acetate-EDTA buffer. Amplification of the actin gene was used as a control for uniform PCR conditions. The intensity of the bands from sRT-PCR amplification was quantified by densitometry using ImageQuant 5.2 software (Molecular Dynamics), and normalized –P/+P expression ratios were obtained.

Quantification of transcripts by qRT-PCR was done by the one-step assay using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad). Assays were done in 25 μL of reaction volume, which contained 12.5 μL of 2× Master Mix, 100 nM forward primer, 100 nM reverse primer, 100 ng of RNA template, and 0.5 mL of iScript reverse transcriptase for one-step RT-PCR. DNase-RNase-free water was used to adjust the volume to 25 μL. Real-time one-step RT-PCR was performed in a 96-well format using the iQ5 Real-Time PCR Detection System and iQ5 Optical System Software (Bio-Rad). The thermal cycler settings for real-time one-step RT-PCR were as follows: 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 59.5°C. Each real-time one-step RT-PCR assay had a melt curve analysis consisting of 80 cycles of 1 min at 95°C, 1 min at 55°C, and 10 s at 55°C, increasing each by 0.5°C per cycle. For each reaction, a product between 100 and 280 bp could be visualized on an agarose gel. Each assay included at least two no-template controls, in which RNA was substituted by DNase-RNase-free water; no amplification was obtained for no-template controls. Quantification was based on a cycle threshold value, with the expression level of each gene in –P nodules as compared with +P nodules normalized by the ubiquitin gene calculated. The sequences of oligonucleotide primers and conditions used in sRT-PCR and qRT-PCR are shown in Supplemental Table S5.

TF profiling, based on real-time qRT-PCR, was performed at the Max Planck Institute of Molecular Plant Physiology to determine nodule differential expression of TF genes. The identification of a set of 372 bean TF genes,

and the design and synthesis of RT-PCR primers for each gene, have been reported (Hernández et al., 2007). Total RNA for qRT-PCR was isolated from 200 mg of frozen nodules as reported (Hernández et al., 2007). Three biological replicas were isolated for each treatment (–P and +P nodules), extracting RNA from different sets of plants grown in similar conditions. RNA concentration was measured in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and 10 mg of total RNA was used for qRT-PCR analysis. Genomic DNA degradation, cDNA synthesis, and quality verification for qRT-PCR were done as reported (Hernández et al., 2007). Quantitative determinations of relative transcript levels of TF genes using RT-PCR were carried out according to Czechowski et al. (2004) and Hernández et al. (2007). The bean phosphatase gene (TC3168) was included as a marker for P deficiency in every qRT-PCR run, using the reported primers (Hernández et al., 2007). TF expression was normalized to that of UBC9, which was the more constant of the four housekeeping genes included in each PCR run. –P/+P average expression ratios were calculated as reported (Hernández et al., 2007). Student's *t* test was performed with a *P* value cutoff of 0.05. Data from statistically differentially expressed ESTs with a –P/+P expression ratio of 2 or more were analyzed as mentioned below.

Plant Metabolite Extraction

Plant metabolite extraction of nodule samples from –P- and +P-treated bean plants and GC-MS metabolite profiling were done as reported previously (Colebatch et al., 2004; Desbrosses et al., 2005; Hernández et al., 2007). Twelve replicate samples for each condition, namely nodules from plants grown under +P and –P conditions, were harvested at 21 dpi from pods, rinsed with tap water, dried on filter paper, and shock frozen in liquid N. Frozen samples of 35 to 70 mg fresh weight were ground by mortar and pestle under liquid N in order to keep samples metabolically deactivated. Frozen powder was [AQ10] extracted with hot methanol/$CHCl_3$, and the fraction of polar metabolites was prepared by liquid partitioning into water. Further processing was as described by Desbrosses et al. (2005).

GC-Time of Flight-MS Metabolite Profiling

GC-time of flight (TOF)-MS profiling was performed using a FactorFour VF-5ms capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) with a 10 m EZ-guard precolumn (Varian) and an Agilent 6890N gas chromatograph with splitless injection and electronic pressure control mounted to a Pegasus III TOF mass spectrometer (LECO Instrumente). Details of the GC-TOF-MS adaptation of the original profiling method (Desbrosses et al., 2005) are described by Wagner et al. (2003) and Erban et al. (2006). Metabolites were quantified as relative changes of pool sizes after mass spectral deconvolution (ChromaTOF software version 1.00, Pegasus driver 1.61; LECO) of at least three mass fragments representing each analyte. Peak height representing arbitrary mass spectral ion currents of each mass fragment was normalized using the amount of the sample fresh weight, and ribitol was added as an internal standardization to correct for volume variations. Normalized responses (g^{-1} fresh weight) and response ratios were calculated as described (Colebatch et al., 2004; Desbrosses et al., 2005).

Identification of Metabolites within GC-MS Metabolite Profiles

Metabolites were identified using the NIST05 mass spectral search and comparison software (National Institute of Standards and Technology; http://www.nist.gov/srd/mslist.htm) and the mass spectral and retention time index (RI) collection (Schauer et al., 2005) of the Golm Metabolome Database (GMD; Kopka et al., 2005). Mass spectral matching was manually supervised, and matches were accepted with thresholds of match > 650 (with maximum match equal to 1,000) and RI deviation $< 1.0\%$ (for details, see Table II; Supplemental Table S6). Information on the polar metabolites, using the corresponding mass spectral identifiers, can be found at http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_smq.html. Metabolites are characterized by Chemical Abstracts System identifiers and compound codes issued by KEGG (Kanehisa et al., 2004). Metabolites were identified by standard substances or by as yet unidentified MSTs of GMD. The term MST is used for repeatedly occurring but nonidentified compounds, which can be recognized by mass spectrum and RI as defined earlier (Colebatch et al., 2004; Desbrosses et al., 2005). MSTs are tentatively characterized and named by best mass spectral match to compounds identified by NIST05 or GMD using match value and hit name (Table II; Supplemental Table S6). The response ratio –P/+P for each metabolite/MST was calculated by dividing the average metabolite concentration from 12 nodule samples of P-deficient plants by the average metabolite concentration from 12 nodule samples from roots of control plants (Table II; Supplemental Table S6).

ICA and Statistical Analysis

ICA (Scholz et al., 2004) was applied to metabolite profiles (as compiled in "Supplemental Data"). Data were normalized by calculation of response ratios using the median of each metabolite as the denominator and subsequently subjected to logarithmic transformation ($\log_{10}$). Missing value substitution was as described earlier (Scholz et al., 2005). Statistical testing was performed using Student's *t* test. Logarithmic transformation of response ratios was applied to better approximate the Gaussian normal distribution of metabolite profiling data required for statistical analyses.

Data Analyses

Three bioinformatics-based approaches were used for analyses aimed to interpret the biological significance of gene expression data in combination with metabolome data.

First, we aimed to detect whether a certain category, as defined by the UniProt keywords, was statistically overrepresented in the differentially expressed sets of ESTs (induced or repressed in –P) compared with the rest of the ESTs. For this, the *P* value for all UniProt keywords was calculated using the hypergeometric distribution, as described in GeneBins (Goffard and Weiller, 2007a; Supplemental Table S4).

A second approach for expression data analysis was based on MapMan software version 2.2.0 (Thimm et al., 2004; http://gabi.rzpd.de/projects/MapMan/). In order to extend MapMan to common bean, a soybean (*Glycine max*) mapping developed by S. Yang (University of Minnesota; unpublished data) was uploaded to MapMan. Soybean genes homologous to common bean differentially expressed genes were manually identified by BLASTN comparisons with the soybean consensus sequences (http://www.affymetrix.com/products_services/arrays/specific/soybean.affx), and the Affymetrix-Gm identifier for each homologous gene was retrieved. To verify if the putative bean and soybean homologous genes indeed had the same gene annotation, each retrieved Affymetrix-Gm identifier was submitted to the soybase Affymetrix-Soybean Genome Array Annotation Version 2 Page (http://www.soybase.org/AffyChip/), and only the genes with similar annotation in bean and soybean were considered for MapMan submission. The expression ratio –P/+P of the induced or repressed bean genes, expressed in $\log_2$, in combination with the list of Affymetrix-Gm identifiers were used to visualized the common bean gene expression data. Supplemental Table S7 shows the complete list of bean genes submitted to MapMan with their homologous soybean gene identifiers and the expression ratios.

The third type of analysis used the PathExpress Web-based tool (Goffard and Weiller, 2007b; Goffard et al., 2009) in order to identify the most relevant metabolic pathways associated with the subsets of differentially expressed genes. PathExpress was extended to bean. The data used to build the bean metabolic network were derived from the current release of the KEGG LIGAND database (release 42.0; Kanehisa et al., 2004). If the best match in UniProtKB for all ESTs from the bean nodule and root cDNA libraries had been annotated as enzymes, each EST was assigned to the corresponding EC number. The two sets of ESTs corresponding to nodule –P-induced or -repressed genes were separately submitted to PathExpress and were compared with the list of all bean enzymes involved in the annotated pathways. The results allowed detection of those ESTs associated with metabolic pathways or subpathways that were statistically overrepresented ($P \leq 0.05$) in the differentially expressed sets of ESTs. The graphs of significant metabolic pathways generated by PathExpress were manually checked in order to identify induced or repressed nodule metabolites participating in those pathways.

Sequence data for this article can be found in the GenBank/EMBL data libraries under accession numbers GO355314 to GO355395.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Differentially expressed genes common to roots and nodules from P-deficient bean plants.

Supplemental Table S1. Genes induced in nodules of P-deficient plants identified by macroarray analysis.

Supplemental Table S2. Genes repressed in nodules of P-deficient plants identified by macroarray analysis.

Supplemental Table S3. Annotation of ESTs from the root and nodule cDNA libraries (DFCI PhvGI) from common bean.

Supplemental Table S4. Total ESTs from bean nodule and root cDNA libraries and ESTs from differentially expressed sets assigned to a certain UniProtKB keyword.

Supplemental Table S5. Primers and conditions used for sRT-PCR and qRT-PCR.

Supplemental Table S6. Complete metabolic profile response from common bean roots.

Supplemental Table S7. Soybean genes homologous to differentially expressed common bean ESTs used for MapMan analysis.

Supplemental Table S8. Nodule transcript levels of all of the genes in the common bean root and nodule macoarrays.

Supplemental Table S9. Nodule transcript levels of all common bean TF genes determined by qRT-PCR.

[AQ11]

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· **Invited Review** ·

Transcriptional Regulation and Signaling in Phosphorus Starvation: What About Legumes?

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Abstract

The availability of soil phosphorus (P), an essential element, is one of the most important requirements for plant growth and crop production. The morphological and physiological adaptations evolved by plants to cope with P starvation have been well characterized. Several P deficiency plant responses are regulated at the transcriptional level. Microarray analysis has generated valuable information on global gene expression in *Arabidopsis thaliana* grown under P-stress. Despite the identification of P responsive genes, little is known about the regulation of gene expression changes. Four transcription factors, PHR1, WRKY75, ZAT6 and BHLH32, involved in P starvation signaling have been characterized in *Arabidopsis*, and signaling pathways are deciphered. This review analyzes the current knowledge of transcriptional regulation of P starvation responses in *Arabidopsis vis-à-vis* legumes such as lupine, common bean and *Medicago truncatula*. The knowledge on regulatory and signaling mechanisms involved in P acquisition and use in legumes will be useful for improvement of these crops, which account for a large proportion of the world's crop production, providing good nutritional quality feed and food.

Key words: legumes; phosphorus starvation responses; P starvation signaling pathways; transcriptional regulation; transcription factors.

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Phosphorus (P) is a vital macro-nutrient for plant growth and development. P is required for essential metabolic processes including energy transfer, signal transduction, biosynthesis of macromolecules, photosynthesis and respiration. Though P is an abundant element of the Earth's crust, it associates with cations (iron or aluminum) or organic compounds forming insoluble complexes that are not accessible to plants. Therefore, more than 30% of the world's arable lands require externally applied P fertilizers for crop production. However, excessive use of fertilizers results in pollution of surrounding lakes and other water bodies leading to environmental degradation. Moreover, chemical fertilizers are manufactured using non-renewable petroleum resources, which will be depleted in no more than 60 years (Vance 2001; Vance et al. 2003).

Plants have evolved morphological, physiological and molecular adaptations to cope with P starvation. The morphological responses involve the modification of root architecture, principally by decreasing primary root growth and increasing lateral root and root hair formation. The physiological and biochemical responses include: modifications of carbon metabolism to bypass P requiring steps, synthesis and secretion of acid phosphatases (AP), exudation of organic acids, and enhanced expression of high affinity phosphate transporters (for reviews, see Raghothama 1999, 2000; Smith 2001; López-Bucio et al. 2003; Vance et al. 2003; Plaxton 2004; Shulaev et al. 2008).

Legumes account for 27% of the world's major crop production, with grain legumes providing more than one-third of humankind nutritional nitrogen requirements. The ability of many grain legumes to establish symbiosis with nitrogen-fixing

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bacteria reduces their cost of cultivation and makes them a valuable source of soil nitrogen for other crops. In sustainable agriculture legumes play a significant role in effective management of fertilizer, improving soil health. However, P deficiency and acid soil conditions form important constraints for legume crop production, especially in Latin America, Asia and Africa. The main focus of P stress research in legumes has been in white lupine (*Lupinus albus*) and common bean (*Phaseolus vulgaris*), and to a lesser extent in *Medicago truncatula* (a model legume system) and soybean (*Glycine max*) (Vance 2001; Brougthon et al. 2003; Graham and Vance 2003; Vance et al. 2003; Tesfaye et al. 2007).

White lupine is characterized by its extreme tolerance to P deficiency that is correlated with a highly coordinated modification of root physiology and biochemistry resulting in the development of proteoid (cluster) roots (Johnson et al. 1996; Keerthisinghe et al. 1998). Unlike typical lateral roots, proteoid roots develop laterals that emerge from every xylem pole within the axis, accompanied by extensive root hair growth, resulting in a more than 100-fold increase in surface area. The physiological role of proteoid roots is the secretion of organic acids for P remobilization (Johnson et al. 1996; Keerthisinghe et al. 1998).

Modified root architecture in response to P stress has been well characterized in common bean. Bean responses to P deficiency include the modification of root growth axis and gravitropism and the formation of shallower and adventitious roots, which facilitate exploration for P resources in the top soil (Bonser et al. 1996; Liao et al. 2001; Lynch and Brown 2001). In an attempt to understand the genetic nature of these root traits, several quantitative trait loci (QTLs) were identified. Some QTLs for root architecture showed a good correlation with P acquisition, something that strengthens the importance of root architecture in bean P deficiency adaptation (Beebe et al. 2006; Ochoa et al. 2006).

Most of our understanding of plant adaptation to P deficiency comes from research in the model system *Arabidopsis thaliana*, and little information is available for other plants such as legumes. Several P deficiency plant adaptation responses are regulated at the transcriptional level with a highly coordinated gene expression program (Raghothama 1999; Franco-Zorrilla et al. 2004). Recently macro/microarray studies have identified genes differentially regulated by P starvation. Despite the identification of P responsive genes, little is known about the regulation of gene expression changes. Transcription factors (TFs) are master control proteins and in *Arabidopsis*, the expression of several TFs is regulated in a cell- or tissue-specific manner in response to specific environmental biotic and abiotic stresses (Chen et al. 2002). The genetic demonstration of the participation of specific TF genes in the signaling pathways for P stress response is beginning to emerge. This work reviews the current knowledge on the global transcriptional regulation P starvation responses, best known for *Arabidopsis* and compares it to less understood process in legumes.

Transcriptional Profile in Response to P Deficiency

The transcriptional profile analyses by microarrays or Affimetrix gene chips have provided valuable information on global changes in gene expression in response to P starvation in *Arabidopsis* roots, shoots and leaves (Hammond et al. 2003; Wu et al. 2003; Misson et al. 2005; Müller et al. 2007). The analysis of the data on kinetics of global gene expression in P-starved *Arabidopsis* (Hammond et al. 2003; Wu et al. 2003; Misson et al. 2005) evidenced the existence of two transcriptional programs: an early program observed between 3 and 72 h and a late program evidenced after 7 d. Table 1 presents representative genes from different functional categories that are induced during early and/or late transcriptional programs.

Once the intracellular P resources are depleted, the initial *Arabidopsis* responses are considered to be general or non-specific for P stress (Franco-Zorrilla et al. 2004). The general responses of the early program include the induction of genes concerned with oxidative stress and pathogen responses (Table 1). Some of these genes are induced only at early stages and their expression decreases after prolonged P stress. Examples of early induced genes that are specific for P starvation adaptation include monogalactosyl diacyl glycerol synthase and digalactosyl diacyl glycerol synthase genes (Table 1) which are responsible for galactolipid synthesis leading to cytoplasmic membrane turnover. Several early-induced genes trigger the programs for determinate root growth required for the modification of root architecture (Table 1). Analysis of the *Arabidopsis* mutants *lpr1* and *lpr2* (low phosphate root) revealed that the induction of these genes, which encode for multicopper oxidases, at the root tip are required to arrest and reprogram root growth, leading to the modification of root architecture upon exposure to low P (Svistoonoff et al. 2007).

During the early P starvation program several genes are repressed, such as genes coding for enzymes involved in nitrogen assimilation (glutamine synthase and glutamate synthase), genes whose products are involved in carbon metabolism (glyceraldehyde 3-phosphate dehydrogenase), in biogenesis of phospholipids (phosphoethanolamine N-metyl transferase), in photosystem I/II (PSI/PSII) and in the Calvin cycle, as well as energy carriers for photosynthetic and mitochondrial electron transport (ferredoxin NADPH reductase). The downregulation of these genes is relevant since their products are involved in regulatory steps for excessive use of cytosolic P (Wu et al. 2003; Misson et al. 2005).

The late transcriptional program mainly includes the genes that are normally involved in the specific responses to P deficiency. These genes are either expressed in the late stage or show gradual induction during P deficiency (Hammond et al. 2003; Wu et al. 2003; Misson et al. 2005), and includes the phosphoenol pyruvate carboxylase and malate dehydrogenase genes that participate in glicolytic/tricholoroacetic acid (TCA)

Anexo VII.4

Table 1. P starvation induced genes

Functional categories and genes	Designation	Gene induced in
Carbon metabolism		
Phosphoenol pyruvate carboxylase	*PEPc*	At (L) La
Malate dehydrogenase	*MDH*	At (L) La
Glyceraldehyde-3-phosphate dehydrogenase	*G-3-P*	La Pv
Pyrophoshate depended phosphofructokinase	*PPi-FructK*	At(L) La Pv
Secondary metabolism		
Cytochrome P450	*CytP450*	At (E, L) La Pv
P remobilization		
Ribonuclease H	*RNS*	At (E, L) La Pv
Purple acid phosphatase 5	*APC5*	At (L) La Pv
Acid phosphatase	*AP*	At (E, L) La Pv
P transport		
Phosphate transporter high affinity 1;4	*PHT1;4*	At (L) La Pv
Phosphate transporter high affinity 3;2	*PHT3;2*	At (L)
Phosphate transporter 1; H1	*PHO1; H1*	At (L) Pv
Phosphate transporter 1; H10	*PHO1; H10*	At (L) Pv
Stress and defense		
Pathogenesis-related protein	*PR*	At (E, L) Pv
Senescence-associated protein	*Senescence-associated protein*	At (E, L) La Pv
Gluthatione peroxidase	*GPX*	At (E, L) Pv
Cu/Zn superoxide dismutase	*Cu/Zn SOD*	At (L)
Catalase	*CAT*	At (E, L) Pv
Peroxidase	*PX*	At (E, L) Pv
Gluthation-S-transferase	*GST*	At (E, L)
Heat shock induced protein	*DNAJ*	At (E, L) Pv
Development and root architecture related genes		
Proline-rich-family protein	*PRP*	At (L) La Pv
Indole-3-acetic acid –induced protein ARG2	*IAAIP*	At (E, L) La Pv
1-aminocyclopropane-1-carboxylate oxidase	*ACCO*	At (L) La Pv
Auxin efflux carrier	*AEC*	At (E, L)
Subtilisin-like-serine protease	*AIR3*	At (E, L)
Allergen-like protein	*ALP*	At (E, L)
Extensin	*Extensin*	At (E, L) La Pv
Low phosphate root 1	*LPR1*	At (E, L)
Low phosphate root 2	*LPR2*	At (E, L)
P homeostasis		
At4	*At4*	At (L)
microRNA399	miR399	At (L)
Phospholipids biosynthesis		
Phospholipase D	*PLD*	At (E, L)
Phospholipase C	*PLC*	At (L)
Monogalactosyl diacyl glycerol synthase	*MGDG*	At (E, L)
Digalactosyl diacyl glycerol synthase	*DGDG*	At (E, L)
Sulfhoquinovosyl diacyl glycerol	*SQDG*	At (L)
Anthocyanin biosynthesis		
Anthocyanidin synthase	*ANS*	At (L)
Flavone-3-hidroxylase	*Flavone-3-hidroxilase*	At (L)
Chalcone synthase	*CHS*	At (L)

Table 1. Continued.

Functional categories and genes	Designation	Gene induced in
Transcription regulation		
Zin Finger C3HC4 type RING	*C3HC4*	At (E)
WRKY	*WRKY*	At (E, L) Pv
WRKY1	*WRKY1*	At (E) Pv
AP2	*AP2*	At (E, L)
MYB	*MYB*	At (L) La Pv
MYB-CC	*PHR1*	At (L)
WRKY75	*WRKY75*	At (E, L)
C2H2	*ZAT6*	At (E, L)
TIFY	*TIFY*	Pv

Two letter designators are used to describe the species that show P-starvation induction for each gene: At refers to *Arabidopsis thaliana* early (E) or late (L) transcriptional programs, La refers to *Lupinus albus* (lupine) and Pv refers to *Phaseolus vulgaris* (bean). Information for this table was extracted from: Hammond et al. (2003); Uhde-Stone et al. (2003a,b); Wu et al. (2003); Misson et al. (2005); Bari et al. (2006); Graham et al. (2006); Shin et al. (2006); Chen et al. (2007); Franco-Zorrilla et al. (2007); Hernández et al. (2007); Müller et al. (2007); Stefanovic et al. (2007); Svistoonoff et al. (2007); Tian et al. (2007).

pathways and whose products promote the synthesis of organic acids that are secreted for P remobilization in the soil, the genes involved in P remobilization (phosphatases, RNAses), P transport, anthocyanin biosynthesis and modification of root architecture (Table 1). Genes related to oxidative stress, such as Cu/Zn superoxide dismutase and glutathione peroxidase, are also induced specifically during the late stages of P starvation (Table 1). In addition, non-specific P responsive genes, such as the *DNAJ*, pathogenesis- and senescence-related genes, are also expressed during the late transcriptional program (Table 1). Analysis of the *Arabidopsis* early and late transcriptional programs revealed induced genes that are common for other abiotic stresses. This suggests that the crosstalk between different abiotic stress pathways is necessary for plant survival.

Several genes are specifically repressed in the late program, such as some genes implicated in protein synthesis, in P homeostasis (ubiquitin E2 conjugase), in iron homeostasis and transport, and in transcriptional regulation (homeobox-leucine zipper, CCHC-type zinc finger) (Wu et al. 2003; Misson et al. 2005). An important role of the late program repressed genes is to maintain the P homeostasis and to regulate cytosolic P use.

In P-starved *Arabidopsis* plants, P content of the roots and shoots decreases to 50% as compared with P sufficient conditions, whereas the P content in leaves only decreases by 10%. However, more than 50% of P responsive genes are greatly induced in leaves compared with other organs. This behavior suggests that each organ has different strategies for P sensing and for coping with P stress (Wu et al. 2003; Misson et al. 2005).

Bioinformatic analyses of expressed sequence tag (EST) databases enable the identification of candidate abiotic stress-induced genes, and provide complementary data to the microarray studies. Datasets of ESTs responsive to P-deficiency have been developed for four legumes: *Medicago* roots and leaves, soybean (*Glycine max* and *Glycine sojae*) and common bean roots (Dana Farber Cancer Institute, http://compbio.dfci.harvard.edu/tgi/), and lupine proteoid roots (Uhde-Stone et al. 2003a,b). Taking advantage of this information, Graham et al. (2006) presented bioinformatic analysis of genes that mediate adaptation to P starvation in the above-mentioned legume species and *Arabidopsis*. Clustering analyses across five species led to the identification of 22 groups of genes, from different functional categories that are statistically over-represented in EST contigs from P-stressed tissues of five or of four of the species studied (Graham et al. 2006). In addition, 52 bean candidate genes, belonging to 19 categories (transport, stress, signal transduction) were identified as being induced by P deficiency (Graham et al. 2006). However, genes involved in P transport, sulfo- and galacto-lipids biosynthesis and organic acid biosynthesis were not identified in this *in silico* analysis. The latter is probably because the bean EST library was prepared from the late stage P starved roots (21 d), or because the analysis was restricted only to the ESTs assigned to contigs as singletons were not considered (Graham et al. 2006).

White lupine, a highly P stress-tolerant legume, is a good model to decipher the mechanisms for the adaptation to P starvation. In an attempt to better understand the molecular events mediating the white lupin adaptation to P deficiency, using high density macroarray, Uhde-Stone et al. (2003a,b) reported the transcriptional profile of proteoid roots grown under P deficiency. Most of the genes induced in proteoid root development under P starvation were found to be involved in the metabolic bypassing of phosphate use (PPi-dependent phosphofructokinase, G3P), organic acid biosynthesis (PEPc, MDH), P remobilization, phytohormone metabolism, and proteoid root development (Table 1; Uhde-Stone et al. 2003a,b). In white lupine, only the cytokinin

oxidase gene, which is involved in cytokinin degradation, was repressed in P-starved proteoid roots.

Two different approaches, namely macroarrays and suppressive subtractive cDNA library analyses, have been used for deciphering global gene expression in response to P deficiency in common bean (Hernández et al. 2007; Tian et al. 2007). The analysis of a suppressive subtractive cDNA library was used to identify P stress-induced genes in 10-d-old bean roots from P-deficient plants. The genes identified (240) are found to be mainly involved in the modification of carbon metabolism and photosynthesis to bypass P-requiring steps, and P transport (Table 1; Tian et al. 2007). High-density macroarrays, printed with ESTs derived from a P-deficient bean root cDNA library (Ramírez et al. 2005), were used to analyze the transcript profile of bean roots after 21 d of P-starvation. In this work, 126 P responsive genes representing different functional categories, such as secondary metabolism, regulation/signal transduction and genes encoding proteins that participate in intracellular and extracellular transport were identified (Table 1). Similarities in both analyses refer to induced genes that mediate P remobilization and stress/defense processes. In agreement, the non-biased metabolic profile of bean roots revealed that stress-related metabolites such as polyols and proline are accumulated in P-deficient treatment (Hernández et al. 2007). Unlike in *Arabidopsis*, genes implicated in sulfo- and galactolipids and anthocyanin biosynthesis were not identified among P responsive genes in bean. This could be explained by the fact that the bean transcript profile has only been analyzed from roots, whereas in *Arabidopsis*, induction of the genes involved in such processes were analyzed mostly from leaves (Misson et al. 2005). Although genes involved in organic acid synthesis were not identified in P stressed bean plants, the metabolic profile revealed a reduced amount of some of the organic acids in P-deficient bean roots, which likely reflects exudation from the root into the rhizosphere (Hernández et al. 2007; Huang et al. 2008). Though the bean transcript profile analysis suggests that different early and late responses are operating, the existence of different transcriptional programs similar to those of *Arabidospsis* remains to be demonstrated.

Sugars, apart from being metabolites, are also recognized as signal molecules in plants. In *Arabidopsis* and lupine, it has been observed that sugars and P stress signaling are closely interrelated (Franco-Zorrilla et al. 2004; Liu et al. 2005; Karthikeyan et al. 2006; Müller et al. 2007). Transcript profile of *Arabidopsis* leaves contrasting in combinations of P and carbohydrate levels revealed interactions between P- and sugar-dependent gene regulation (Müller et al. 2007). In addition, *Arabidopsis* mutants impaired in sugar transport or signaling showed reduced induction of P responsive genes, and the affected phenotype could be rescued by the addition of sugars (Karthikeyan et al. 2006). Moreover, common DNA sequences for TF binding have been found in the promoters of genes that respond to sugar or P stress (Müller et al. 2007). Similarly, lupine research supports the interrelation between P and sugars signaling. The expression of three P-stress induced lupine genes (*LaPT1*, *LaSAP1*and *LaMATE*) decreases in dark-grown or stem-girdled P-starved lupine plants and this is rescued by sugar addition (Liu et al. 2005). However, these results do not exclude the requirement of the transport of other signal molecules.

Signaling Pathways in Response to P Starvation

Despite the information derived from macro/microarray analyses on global gene expression in response to P-starvation, little is known about the regulation of gene expression changes. TFs are master control proteins and regulate gene expression in response to different stimuli. TFs bind to DNA and interact with other regulatory proteins, including the proteins for chromatin remodeling/modification, to recruit or block access of RNA polymerase to the DNA template. Approximately 7% of plant genomes code for TFs and in *Arabidopsis* this accounts for more than 1 800 genes that have been classified into different gene families. However, in *Arabidopsis* not more than 10% of functions of TFs have been delineated (Riechmann and Ratcliffe 2000).

Chen et al. (2002) reported that some of the *Arabidopsis* TF genes are regulated transcriptionally in response to specific environmental biotic and abiotic stresses. The expression of 402 TF genes was analyzed in different organs, developmental stages and stress conditions. The same TF genes were expressed in several of the conditions analyzed, indicating the complexity of regulatory networks (Chen et al. 2002). Wu et al. (2003) reported that approximately one third of the 333 TF genes included in the *Arabidopsis* microarray were differentially expressed under P-starvation conditions. On the other hand, Misson et al. (2005) and Müller et al. (2007) reported some 80 TF genes that respond to P stress in *Arabidopsis*. The P-responsive TFs identified belong to different gene families, comprising MYB, SCARECROW, AP2, F-box, HOMEOBOX, WRKY and Zinc-finger members (Wu et al. 2003; Misson et al. 2005; Müller et al. 2007).

It is worthwhile noting that in order to demonstrate the involvement of a particular TF in the regulation of the response to a certain abiotic stress, the use of genetic approaches is imperative, and thus the association of gene induction with a specific abiotic stress is not enough evidence. The phenotypic characterization of different *Arabidopsis* mutants has shown the involvement of four TF genes: *AtPHR1*, *AtWRKY75*, *AtZAT6* and *AtBHLH32*, in signaling during the P stress response. Figure 1 is a diagrammatic representation of the intricate signaling pathways controlled by these four TFs.

The *Arabidopsis* MYB TF, PHR1, was the first TF to be identified in vascular plant P stress signaling (Rubio et al. 2001). Orthologs from rice (*OsPHR1* and *OsPHR2*) with similar

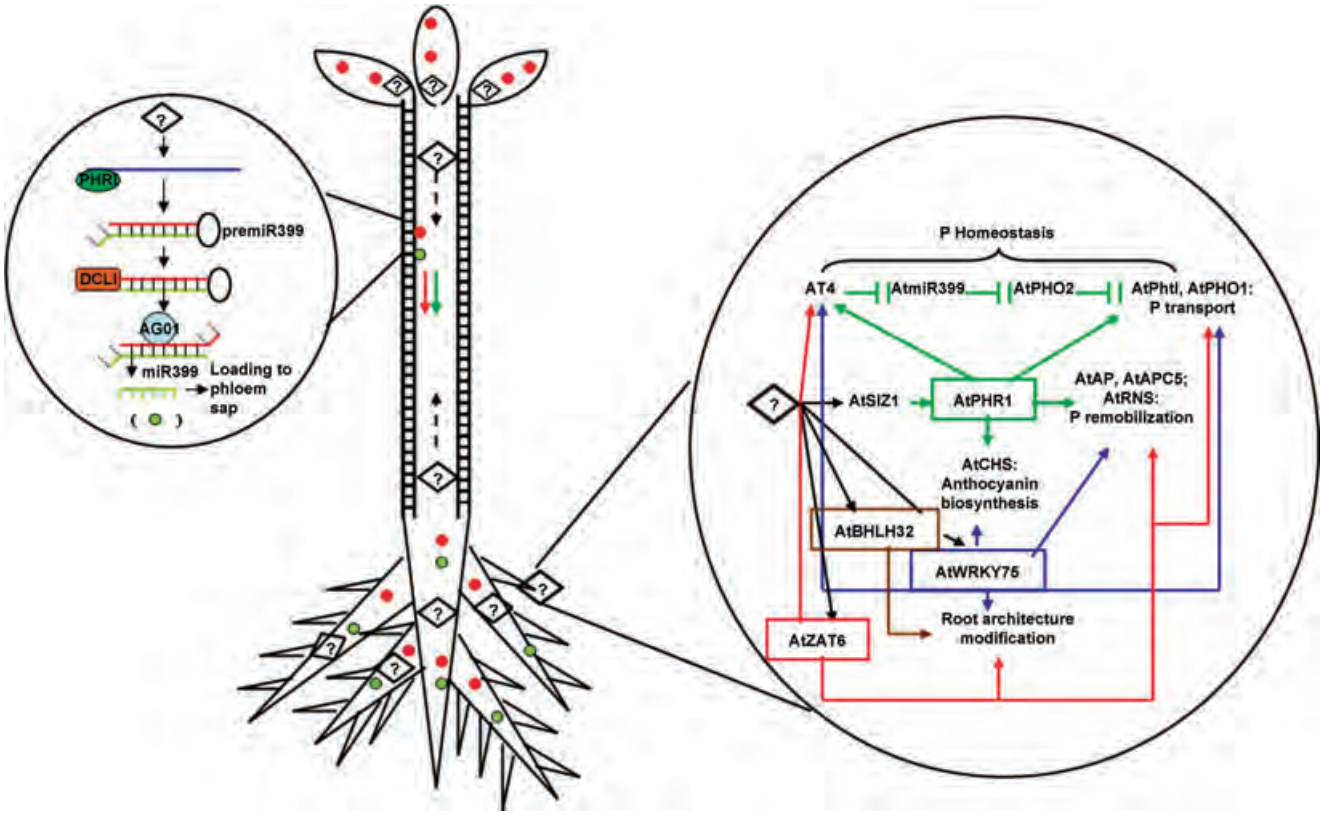

Figure 1. Model for P starvation signaling pathways in *Arabidopsis thaliana.*

Signals synthesized in the shoots are translocated to the roots: sugars (red circle), derived from photosynthate, and mature miR399 (green circle), that is processed as represented in the left circle. Left circle: miR399∗ (red line), miR399 (green line), DCL1: dicer-like 1, AGO1: argonaute protein. Unknown P starvation sensor/signal molecule (? in rhombus). Right circle: the unknown sensor/signal molecule(s) transduces the signal to each of the four P starvation TF (in rectangles). The signaling pathway directed by each transcription factor (TF) is represented as PHR1 (green), WRKY75 (blue), ZAT6 (red) and BHLH32 (brown). Arrows indicate positive regulation and bars indicate negative regulation.

functions in P stress signaling have recently been characterized (Zhou et al. 2008). Features of the *Arabidopsis* PHR1 signaling pathway are shown in Figure 1. P deficiency is sensed either locally or systemically by an unknown molecule(s). PHR1 is nuclear localized through sumoylation by a SUMO E3 ligase (SIZ1) (Rubio et al. 2001; Misson et al. 2005) which recognizes the imperfect palindrome GNATATNC that is present in the promoter regions of the genes involved in P remobilization (*APs* and *RNS*), transport (*PTH1, PHO1; H1* and *PHO1; H10*) and homeostasis (*At4*, miR399), as well as genes for anthocyanin biosynthesis (Rubio et al. 2001; Nilsson et al. 2007; Stefanovic et al. 2007; Ribot et al. 2008). Three regulatory genes (*At4*, miR399 and *PHO2/UBC24*) of the PHR1 signaling pathway that are relevant for the regulation of P homeostasis have been characterized: *At4* is a P-starvation induced gene from the *IPS1* gene family, and is positively regulated by PHR1. This and other genes from that family lack a long open-reading frame (ORF) and instead they contain a series of short overlapping ORFs. Though the function of *At4* is unknown, it plays an important role in the translocation of P from roots to shoots (Burleigh and Harrison 1999; Shin et al. 2006). The precursor of the microRNA 399 (miR399) is positively regulated by PHR1, and is processed by Dicer-Like 1 (DCL1) and argonaute (AGO1) proteins (Jones-Rhoades et al. 2006). Mature miR399 has been proposed as a signal molecule for P starvation. The precursor and miR399∗/miR399 have been detected only in the shoot phloem but not in the roots (Buhtz et al. 2008; Pant et al. 2008). During −P condition, the mature miR399 is translocated through the phloem to the roots where it recognizes and degrades its target gene *PHO2/UBC24*, an ubiquitin E2 conjugase (Fujii et al. 2005; Aung et al. 2006; Bari et al. 2006). The high affinity P transporter PHT1 is a target for PHO2 degradation, so the miR399 mediated PHO2 suppression allows an adequate concentration of PHT1 in the membrane of P-starved plant cells. Additionally, miR399 activity is negatively regulated by *At4* through a target mimicry mechanism, recently described to

occur only in plants (Franco-Zorrilla et al. 2007). A region within the *At4* sequence is complementary to AtmiR399, but the pairing is interrupted by a mismatch loop at the miRNA cleavage site. Thus *At4* is not cleaved but instead sequesters miR399, thus preventing the degradation of its original target gene product, *PHO2*. This elegant mechanism enables the transient down-regulation of PHO2 by miR399 during P starvation, allowing not only a quick response to starvation but also a quick return to normal levels afterwards to prevent P toxicity (Franco-Zorrilla et al. 2007).

Other TFs involved in P deficiency signaling in *Arabidopsis* are WKRY75 and ZAT6, members of WRKY and C2H2 (Zinc finger) TF families, respectively (Devaiah et al. 2007a,b). These TFs are upregulated in P starvation and localized to the nucleus, independently of P status of the plant, albeit their nuclear localization is directed by a different sumoylation process than that for PHR1. WRKY75 recognizes W boxes present in regulatory regions of the genes involved in P remobilization, transport and homeostasis (*At4*). However, it has not been fully established that this TF regulates P homeostasis. ZAT6 regulates several genes of the PHR1 and WRKY75 pathway. In contrast to PHR1, both WRKY75 and ZAT6 are implicated in regulating the modification of root architecture, independent of the P status of the plant (Devaiah et al. 2007a,b; Figure 1).

The fourth TF gene that participates in P starvation signaling in *Arabidopsis* is *BHLH32* (Chen et al. 2007). Contrary to *PHR1, WRKY75* and *ZAT6, BHLH32* is downregulated during P starvation. This TF is also implicated in the modification of root architecture. Moreover, BHLH32 is a negative regulator of PEP-kinase, which is positively regulated by PHR1 and is implicated in the modification of carbon metabolism in response to P stress (Chen et al. 2007; Figure 1).

Interestingly, different TFs are involved in P stress signaling (PHR1, WRKY75 and ZAT6) share common target genes. This behavior suggests plasticity and synergism in different signaling pathways that would allow plants to cope with abiotic stress and to survive in adverse environments.

Bioinformatic analyses based on genomic databases may aid in predicting global sets of TF genes for different plant species. An *in silico* search for genes coding for proteins with previously identified Inter-Pro domains (www.ebi.ac.uk/interpro) characteristic of or diagnostic for TF in the DFCI Common Bean Gene Index v.1.0 (9 484 unigene set) (http://compbio.dfci.harvard.edu/tgi/) led the identification of a set of 372 bean TF genes (Hernández et al. 2007). Based on the classification of *Arabidopsis* TF genes (Riechmann and Ratcliffe 2000), bean TF were grouped into 47 gene families. Though the bean TF set is only partial (since total bean genome sequence information is not available), there is a general correspondence between the most abundant TF families in beans with those from *Arabidopsis* (Hernández et al. 2007). A similar TF gene prediction analysis is in progress for *Medicago*, based on the International Medicago Gene Annotation Group (IMGAG) dataset (Udvardi et al. 2007). From the almost completed genome sequences of the two model legumes, *Medicago truncatula* and *Lotus japonicus* (Sato et al. 2007), more than 2 000 TF genes can be predicted for each plant (Udvardi et al. 2007).

Information on the participation of specific TF in signaling of legume responses to abiotic stress is almost non-existent. To our knowledge there are only two publications concerning the response of TF genes in legume plants grown under abiotic stress. A bZIP TF from tepary bean (*Phaseolus acutifolius)* and common bean, and a TF from the AP2/EREBP family that respond to drought and other abiotic stresses, have been reported (Li et al. 2005; Rodríguez-Uribe and O'Connell 2006). The *in silico* analysis based on legume ESTs libraries (bean, soybean, lupine and *Medicago*) attempting to identify genes over-represented in P starved tissues revealed the annotation of various putative TF such as WRKY, MYB and Zn-finger (Graham et al. 2006).

Hernández et al. (2007) published a study on the global expression of common bean TF genes in response to P deficiency. For this analysis a quantitative real-time RT-PCR (qRT-PCR) platform was built based on the whole set of bean TF genes (372) previously defined through the *in silico* analysis. TFs are among the least induced genes; therefore the qRT-PCR technology has proven to enhance the efficiency, precision and robustness for quantifying differential expression of TF genes as compared with microarray/gene chip approaches (Czechowski et al. 2004; Hernández et al. 2007). The bean TF transcript profile revealed 17 genes differentially expressed in P-starved roots. Of these, four TF genes are induced: three belong to the MYB family and one to the TIFY (previously ZIM) family. The TC2883 MYB TF (DFCI/Common Bean Gene Index v.1.0), was induced twofold in bean P stressed roots, is 63% homologous to *AtPHR1 (PvPHR1)*. Through BLATX analysis in the DFCI/Common Bean Gene Index bean genes orthologs of *Arabidopsis* genes from the PHR1 signaling pathway (Figure 1) such as *PvSIZ1* (SUMO E3 ligase) (TC2445), *PvPHO2* (ubiquitin E2 conjugase) (TC1095), *Pv4* (CV536419), have been identified (Valdés-López et al. unpubl. data, 2008). The possible role of PvPHR1 and other regulatory proteins in P starvation signaling remains to be demonstrated in beans. Hernández et al. (2007) reported TIFY family TF (TC1670) that is induced twofold in response to P starvation in bean roots. This gene is 41% homologous to the *Arabidopsis* TIFY TF denominated *AtJAZ*, which responds to ozone and salinity stresses and is a negative regulator of members of the signal transduction pathway mediated by jasmonic acid (Chini et al. 2007; Thines et al. 2007). The possible role of this TIFY TF in bean signaling to P starvation remains to be analyzed.

The *in silico* clustering analysis by Graham et al. (2006) identified 12 WRKY TFs overrepresented in P starved tissues of soybean, white lupine and bean. Seven putative bean WRKY TF genes are being analyzed by the group of CP Vance at the University of Minnesota/USDA (Vance 2007) to decipher their

possible involvement in P starvation signaling (Tesfaye et al. 2007). Preliminary observations indicate that some members of the WRKY family are induced or repressed in P-limited root tissue of common bean and that these TFs can bind to 5′ upstream promoters of Pi deficiency induced genes (J Liu and CP Vance, unpubl. data, 2007).

Other regulatory genes that participate in legume responses to P starvation include the *Medicago* ortholog of *At4 (Mt4)* that has an important role in P homeostasis in P stressed plants (Burleigh and Harrison 1998, 1999). This gene is induced in roots of *Medicago* P starved plants and it is highly sensitive to repression by phosphate fertilization as well as by colonization with arbuscular mycorrhizal fungi. The promoter region has conserved *cis*-elements found in P starvation inducible genes of yeast, *Arabidopsis* and tomato. The presence of a translocatable shoot factor responsible for mediating the systemic downregulation of the *Mt4* gene product in roots has been postulated (Burleigh and Harrison 1998, 1999).

Perspectives

In this review, an overview of the transcriptional regulation and signal transduction in P deficiency in both *Arabidopsis* and legumes has been provided. As mentioned above, the knowledge about legume signaling pathways in response to P stress is incipient, but there are signs that this situation will improve in the near future. Within the next 2 or 3 years complete or nearly complete genomic sequences of three legumes (*Medicago*, lotus and soybean) will be available (Sato et al. 2007). Genomic sequences will result in better physical and genetic maps that will facilitate *in silico* analyses, map-based cloning and characterization of other TF and regulators that participate in abiotic stress signaling. In addition, reverse-genetics approaches are likely to play a more significant role in functional characterization of components of signal transduction pathways. Some resources for reverse-genetics of legumes are beginning to emerge, including mutant populations of lotus and *Medicago* generated with transposon insertions and with EMS for TILLING, and plant transformation protocols for RNAi silencing and overexpression of specific genes (Udvardi et al. 2007).

In contrast to *Arabidopsis*, legumes are not easily amenable to stable genetic transformation and hence, protocols for high throughput generation of transgenic legume plants are not available. On the other hand, alternatively, fast, reproducible and efficient protocols for the generation of “composite plants” have been established for the legume species *Medicago*, lotus (*Lotus corniculatus* and *L. japonicus*), soybean and bean (Hansen et al. 1989; Boisson-Dernier et al. 2001; Kumagai and Kouchi 2003; Subramanian et al. 2005; Kereszt et al. 2007; Estrada-Navarrete et al. 2007). Composite plants have untransformed shoots and *Agrobacterium rhizogene*s has mediated transgenic roots. This procedure has proven to be a valuable tool for functional genomics, especially to study the function of genes involved in root biology and root-microbe interactions through the generation of knockdown (RNAi gene silencing) and gain of function composite plants (Kumagai and Kouchi 2003; Subramanian et al. 2005). The “composite plant” approach in conjunction with RNAi silencing technology is being used to study the participation of selected TFs in signaling pathways in P-deficient bean plants.

Based on newly developed genetic and genomic platforms, P stress research is moving toward an exciting phase centered on gene function, the analysis of regulatory plasticity, signal transduction and increased efficiency of P use. The discovery of molecular mechanisms that promote plant acquisition and use of P can be exploited to improve plants by traditional (breeding) or biotechnological approaches. Efforts to obtain improved legume germplasm for P acquisition and utilization will greatly contribute to the practice of economical and environmentally friendly agriculture compatible with increasing requirements for food and feed.

Readers who are interested in this topic are encouraged to also read the paper by Yuan and Liu: “The signaling components involved in plant responses to phosphate starvation” published in the July issue (2008) of JIPB.

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Anexo VII.4

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Essential role of MYB transcription factor: PvPHR1 and microRNA: PvmiR399 in phosphorus-deficiency signalling in common bean roots

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ABSTRACT

Phosphorus (P), an essential element for plants, is one of the most limiting nutrients for plant growth. A few transcription factor (TF) genes involved in P-starvation signalling have been characterized for *Arabidopsis thaliana* and rice. Crop production of common bean (*Phaseolus vulgaris* L.), the most important legume for human consumption, is often limited by low P in the soil. Despite its agronomic importance, nothing is known about transcriptional regulation in P-deficient bean plants. We functionally characterized the P-deficiency-induced MYB TF TC3604 (Dana Farber Cancer Institute, Common Bean Gene Index v.2.0), ortholog to *AtPHR1* (*PvPHR1*). For its study, we applied RNAi technology in bean composite plants. PvPHR1 is a positive regulator of genes implicated in P transport, remobilization and homeostasis. Although there are no reports on the regulatory roles of microRNAs (miRNA) in bean, we demonstrated that PvmiR399 is an essential component of the PvPHR1 signalling pathway. The analysis of *DICER-like1* (*PvDCL1*) silenced bean composite plants suppressed for accumulation of PvmiR399 and other miRNAs suggested that miR399 is a negative regulator of the ubiquitin E2 conjugase: *PvPHO2* expression. Our results set the basis for understanding the signalling for P-starvation responses in common bean and may contribute to crop improvement.

Key-words: abiotic stress; microRNA regulation; transcription factors.

INTRODUCTION

Phosphorus (P) is an essential element for plant growth and development. One of the most important constraints for plant growth is low P availability because of its association with cations (Fe and Al) or organic compounds that create insoluble inaccessible complexes. Plants have evolved several morphological, physiological and biochemical adaptations to cope with P-deficiency (–P) that include mycorrhizal symbiosis, decreased growth rate, modification of root architecture for increased surface area, remobilization of internal inorganic phosphate, modification of carbon metabolism bypassing P requiring steps, synthesis and secretion of acid phosphatases (AP), exudation of organic acids, and enhanced expression of phosphate transporters (for reviews, see Raghothama 1999; Smith 2001; López-Bucio, Cruz-Ramírez & Herrera-Estrella 2003; Vance, Udhe-Stone & Allan 2003; Shulaev *et al.* 2008).

Several P-deficiency adaptation responses are regulated at the transcriptional level (Raghothama 1999; Franco-Zorrilla *et al.* 2004). Microarray/gene chip analyses in *Arabidopsis thaliana* have allowed the identification of differentially expressed genes involved in the plant adaptation to –P (Hammond *et al.* 2003; Wu *et al.* 2003; Misson *et al.* 2005; Morcuende *et al.* 2007; Müller *et al.* 2007). Likewise, phenotypic analysis of different mutants has demonstrated four P-starvation signalling pathways that are controlled by the *AtPHR1*, *AtWRKY75*, *AtZAT6* and *AtBHLH32* transcription factors (TFs) (Rubio *et al.* 2001; Chen *et al.* 2007; Devaiah, Karthikeyan & Raghothama 2007a; Devaiah, Nagarajna & Raghothama 2007b; Nilsson, Müller & Nielsen 2007). The AtPHR1 signalling pathway regulates the expression of genes implicated in P remobilization, transport, homeostasis and anthocyanin biosynthesis (Rubio *et al.* 2001; Nilsson *et al.* 2007). The AtWRKY75, AtZAT6 and AtBHLH32 signalling pathways, in addition to regulating gene expression of some of these processes including carbon metabolism modification, are implicated in regulating changes in root architecture (Chen *et al.* 2007; Devaiah *et al.* 2007a,b).

Besides the transcriptional regulation of adaptation to –P, P homeostasis in *Arabidopsis* is post-transcriptionally regulated by the microRNA 399 (miR399), an essential component of the PHR1 signalling pathway (Fujii *et al.* 2005; Aung *et al.* 2006; Bari *et al.* 2006; Chiou *et al.* 2006). This microRNA (miRNA) is positively regulated by AtPHR1 (Aung *et al.* 2006; Bari *et al.* 2006) and is negatively regulated by

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IPS1/At4 by means of the target mimicry mechanism (Franco-Zorrilla *et al.* 2007). The relevant role of miR399 during P starvation is exerted by promoting the degradation of the *PHO2* (ubiquitin E2 conjugase) mRNA, because PHO2 is a negative regulator of the high-affinity phosphate transporter PHT1 (Fujii *et al.* 2005; Chiou *et al.* 2006).

Common bean (*Phaseolus vulgaris* L.) is the world's most important grain legume for direct human consumption. Environmental factors such as low soil nitrogen and P levels, and acid soil conditions are important constraints for bean production in most areas of Latin America and Africa where the crop is grown (Brougthon *et al.* 2003; Graham *et al.* 2003). Despite the agronomic importance of bean, there is little information on its regulation of P-starvation responses. In an attempt to understand these responses, we have performed a macroarray analysis of –P bean roots from a Mesoamerican bean genotype and identified 124 differentially expressed genes (Hernández *et al.* 2007). Tian *et al.* (2007) identified 240 differentially expressed genes from P-deficient roots of an Andean bean genotype by screening a suppression subtractive hybridization library. Furthermore, using real-time RT-PCR profiling, we identified three MYB TF induced in P-deficient bean roots (Hernández *et al.* 2007). We hypothesized that these induced TF may be involved in the transcriptional regulation of P-deficiency-induced genes.

The aim of this work was to investigate if the common bean MYB TF [TC3604, tentative consensus assigned by the Computational Biology and Functional Genomics Laboratory, Dana Farber Cancer Institute (DFCI), Common Bean Gene Index, v.2.0], orthologous to *AtPHR1*, hereby denominated as *PvPHR1*, is a regulator of the P-deficiency response. We used a novel bean transformation method (Estrada-Navarrete *et al.* 2007) in conjunction with the RNAi gene-silencing technology, utilized for the first time for this species. In order to investigate if miR399 participates in the PvPHR1 signalling pathway, we analysed the expression of P-deficiency response genes in *PvDCL1*-RNAi silenced bean composite plants.

MATERIALS AND METHODS

Plant material and growth conditions

The common bean (*P. vulgaris*) Mesoamerican 'Negro Jamapa 81' cultivar was used in this study. Seeds were surface sterilized and germinated in sterile conditions for 2 d and then planted in pots with vermiculite. Plants were grown in a greenhouse with a controlled environment (26–28 °C, 16 h photoperiod) and were watered with nutrient solution (Summerfield, Huxley & Minchin 1977) with 1 mM (P sufficiency, +P) or 5 μM PO_4 (P-deficiency, –P). Transcript expression analyses were performed in roots and leaves of plants grown for 21 d. Bean composite plants with transgenic roots were generated as reported (Estrada-Navarrete *et al.* 2007, see further discussion). Hairy roots (3–6 cm long) emerging from the *Agrobacterium rhizogenes* infection site were observed during the second week post-infection. After confirming the presence of reporter gene (see further discussion), their normal (untransformed) root system was cut, and composite plants were replanted in pots with vermiculite. These were grown for 21 d under controlled environmental conditions watered with +P or –P nutrient solution, as described previously. After this period, transgenic roots and non-transformed aerial tissues were collected in liquid nitrogen and were stored (–80 °C) until used for phenotypic analyses. Free P contents were determined from different organs of 21-day-old composite plant as reported (Hernández *et al.* 2007). Data from Table 1 represent the average ± standard error of nine replicates from three independent experiments using composite bean plants with high silencing level (80–95%).

Cloning of full-length PvPHR1 cDNA and of mature PvmiR399

PvPHR1 full-length cDNA sequence was cloned based on ESTs partial sequences obtained from nodule and roots cDNA libraries (Ramírez *et al.* 2005), assigned as TC3604 (DFCI/Common Bean Gene Index, v. 2.0). Two primers were designed for *PvPHR1* PCR gene amplification by 5′ rapid amplification of cDNA ends (RACE) (GSP5: CTGCCAC CACAATCTATGTGCTGACCATGA) and 3′ RACE (GSP3: CCGGATTCCTCATCTGATGAAGGGAAAA AGG). The SMART-RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) was used. PCR-amplified cDNA fragments were cloned into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and were sequenced. For overlaps of new sequences SeqManII program in the DNASTAR software package was used (DNASTAR, Inc., Madison, WI, USA). The amino acid sequence alignment between *AtPHR1* and *PvPHR1* was performed using MultAlign software developed by Corpet (1988). The identification of putative sumoylation sites was carried out using the online tools SUMOplot provided by Abgent primary antibody Company (Flanders Court, San

Table 1. Free phosphate concentrations in different organs of *PvPHR1*-RNAi bean composite plants with high silencing level (80–95%) and control composite plants grown under P deficiency

	Control (empty vector) (μmol g^{-1} FW)	*PvPHR1-RNAi* silenced plants (μmol g^{-1} FW)	*P* value
Leaves	2.48 ± 0.131	0.96 ± 0.125	6.44E-06
Stems	3.74 ± 0.516	0.34 ± 0.03	3.33E-05
Roots	1.01 ± 0.029	0.78 ± 0.105	3.87E-02

Data represent the average ± SE of nine replicates from three independent experiments.

Diego, CA, USA) and for predictions of coiled-coil regions, COILS at http://www.ch.embnet.org/software/COILS/form.html was used. The mature miR399 was cloned from a small RNA library of bean seedlings prepared as reported (Elbashir, Lendeckel & Tuschl 2001). This library was used as template for PCR amplification of the AtmiR399 using consensus sequence-specific primers (5′ADmiR399: ACG GAA TTC CTC ACT TGC CAA AGG AGA; 3′ADmiR399: CTG GAA TTC GCG GTT CAG GGC AA) and adaptor sequence primers. The PCR product was cloned in pCR 2.1-TOPO vector (Invitrogen) and was sequenced.

Plasmid construction and plant transformation

In order to obtain the RNAi constructs, we first constructed the pTDT-DC-RNAi vector. This was derived from pBA-DC-RNAi (Jang *et al.* 2007) by replacing the BASTA gene (cut with StuI and BglII) with the tdTomato gene (cut with EcoR1, blunted, and BamHI) from the pRSET-BtdTomato vector (Shaner *et al.* 2004).

The design of primers for gene amplification was based on the reported EST sequences from bean cDNA libraries (Ramírez *et al.* 2005). To generate RNAi constructs, fragments unique to *PvPHR1* (290 bp) or *PvDCL1* (228 bp) coding sequences were amplified using gene-specific forward primers (*PvPHR1*: 5′ACCTGAAAAAGATAAT TGAAGAA; *PvDCL1*: GGATGATGAAAACGGAAAA AGAA) and caccT7 reverse primer (CACCTAATAC GACTCACTARAGGG). The amplified fragments were cloned in the pENTR/SD/D-TOPO vector and were sequenced (Invitrogen). The resulting pENTR-*PHR1* or pENTR-*DCL1* plasmids were recombined into the pTDT-DC-RNAi binary vector. The correct orientation was confirmed by PCR using the WRKY-5-Rev (GCAGA GGAGGAGAAGCTTCTAG) or WRKY-3-Fwd primer (CTTCTCCAACCACAGGAATTCATC) and caccT7 primer (Supporting Information Fig. S1). The resulting pTDT-*PHR1*-RNAi and pTDT-*DCL1*-RNAi plasmids were introduced by electroporation into *A. rhizogenes* K599 and were then used for plant transformation. Bean composite plants were generated as reported (Estrada-Navarrete *et al.* 2007). Putative transgenic hairy roots were confirmed by checking for the presence of red flourescence, resulting from the expression of the tdTomato reporter gene by confocal microscopy. The original root system and all the transgenic roots, except one, were removed to avoid root chimeras, and the composite plants were grown in the greenhouse for 21 d in +P or –P conditions, as described.

RNA extraction and analysis

Total RNA was isolated from 1 g frozen roots or leaves of bean plants and transgenic roots of bean composite plants, grown under similar +P or –P conditions in independent experiments, as reported (Ramírez *et al.* 2005). Isolated RNA preparations were used for semiquantitative RT-PCR (sRT-PCR) and low-molecular-weight RNA-blot analyses. Quantification of transcript levels from the bean genes was performed by two-step RT-PCR using polythymine deoxynucleotide primer following the manufacturer's instructions (Clontech). Bean gene annotation, primer oligonucleotide sequences and sRT-PCR conditions used are shown in Supporting Information Table S1. ^{32}P-radiolabelled probes for miRNAs were synthesized using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) and the primers shown in Supporting Information Table S1. miRNAs were detected in roots of bean plants and in transgenic roots of bean composite plants grown in +P or –P by low-molecular-weight RNA-gel hybridization. Gels, hybridization and washing conditions were performed as reported (Reyes & Chua 2007). The intensity of bands from sRT-PCR amplification or RNA-blot hybridization was quantified by densitometry using the ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA, USA), and the –P/+P expression ratios were obtained.

RESULTS

Identification of putative PHR1 signalling pathway bean genes

We have identified 10 P-starvation-induced common bean genes encoding for putative TF from different gene families (Graham *et al.* 2006; Hernández *et al.* 2007). In this work, we further characterized the TC3604 gene (DFCI/Common Bean Gene Index, v.2.0, http://compbio.dfci.harvard.edu/tgi/) classified in the MYB superfamily and induced twofold in –P roots (Hernández *et al.* 2007). TC3604 is proposed as the bean ortholog of the *AtPHR1* (AJ310799) gene, TF implicated in signalling of the response to P starvation in *Arabidopsis* and rice (Rubio *et al.* 2001; Zhou *et al.* 2008), hence designated *PvPHR1*. The full-length *PvPHR1* cDNA clone (1.38 kb) (accession number EU500763) contained a 984 bp open-reading frame (ORF) with 63% identity to *AtPHR1*. It encodes for a deduced 328 amino acid protein (Mr = 35.63 kDa). The characteristic MYB domain for DNA binding, coiled-coil domain for protein–protein interaction, and three putative sumoylation sites could be identified from the deduced amino acid sequence (Fig. 1a). *PvPHR1* was induced in leaves (twofold) and roots (1.6-fold) of P-deficient bean plants (Fig. 1b).

In *Arabidopsis*, miR399 is part of the AtPHR1 signalling pathway and plays an important role in P homeostasis (Bari *et al.* 2006; Chiou *et al.* 2006). In an attempt to identify miRNAs possibly involved in bean stress responses, we prepared a miRNA library from bean seedlings and identified sequences that were homologous to *Arabidopsis* miRNAs (Arenas-Huertero *et al.*, unpublished data). From this library, we cloned a miRNA identical in sequence to AtmiR399*b* and *c*, hereby designated as PvmiR399 (Supporting Information Fig. S2a). Moreover, an *in silico* analysis of the National Center for Biotechnology Information (NCBI) dbEST led us to identify clone EG594372 as the reverse complement sequence containing the mature PvmiR399. RNA folding of this

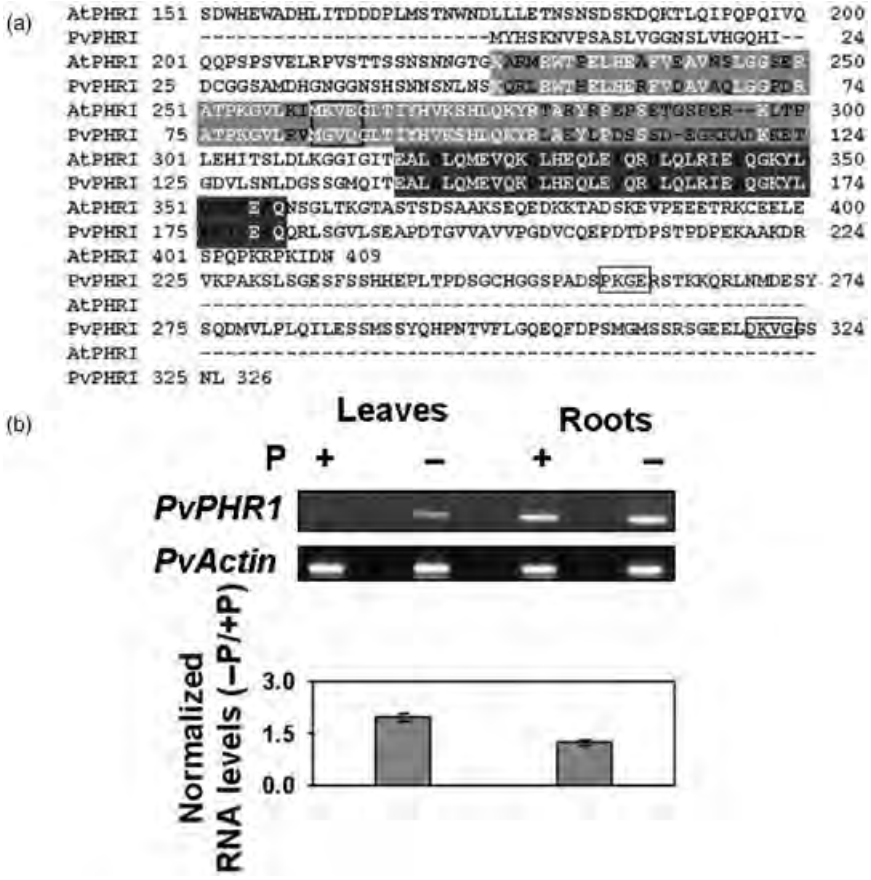

Figure 1. *PvPHR1* cDNA clone. (a) Comparison between *Arabidopsis* PHR1 and PvPHR1 deduced amino acid sequences. Conserved regions corresponding to the MYB domain (light grey), coiled-coil domain (dark grey) and putative sumoylation sites (inside a rectangle) are highlighted. White letters indicate conserved amino acids within each domain. (b) Transcript levels of *PvPHR1* in leaves and roots of P-sufficient (+P) and P-deficient (–P) bean plants were detected by semiquantitative RT-PCR (sRT-PCR) using the actin gene as control for uniform sRT-PCR conditions. The intensity of the bands from amplified products was quantified densitometrically, and the –P/+P normalized expression ratios were obtained. Values are mean ± SE of three biological replicates.

sequence using the mfold program developed by Zuker (2003) is consistent with it being the PvmiR399 precursor (data not shown). PvmiR399 accumulation was highly induced (80-fold) in roots of P-deficient bean plants when compared with control samples under P sufficiency (+P) (Fig. 2a).

In order to investigate the regulatory role of PvmiR399, we searched for bean orthologs of *Dicer-like* (*DCL*) genes implicated in miRNAs biogenesis. Four *DCL* genes involved in the generation of small RNAs have been identified in plants. Specifically, the DCL1 protein is the major player involved in miRNA biogenesis; however, in its absence, it can be partially substituted by DCL2 (Deleris *et al.* 2006; Jones-Rhoades, Bartel & Bartel 2006). Searching the Common Bean Gene Index (DFCI), we identified an EST, CV544176, which has 80% identity to *AtDCL1*, hereby designated as *PvDCL1* (Supporting Information Fig. S2b). Importantly, this gene did not respond to –P treatment (data not shown).

In addition, we identified orthologs of two genes also implicated in P homeostasis: *At4* (TC7206, *Pv4*) and *PHO2* (TC8137, *PvPHO2*) (Aung *et al.* 2006; Shin *et al.* 2006) (Supporting Information Fig. S2c,d). *At4* and its ortholog from the legume *Medicago truncatula* (*Mt4*) belong to the *IPS1* (induced by phosphate starvation) gene family and lack a long ORF, containing instead a series of short overlapping ORFs. These genes play an important role in the translocation of P from roots to shoots (Shin *et al.* 2006). We found a clear induction of *Pv4* in leaves (18-fold) and roots (29-fold) of P-deficient bean plants when compared with +P plants (Fig. 2b). The ubiquitin E2 conjugase *AtPHO2* is a negative regulator of the AtPHR1 signalling pathway (Fujii *et al.* 2005; Chiou *et al.* 2006). In agreement, *PvPHO2* was repressed in leaves (twofold) and roots (1.8-fold) of P-deficient bean plants when compared with +P plants (Fig. 2c).

PvPHR1 participates in P-deficiency signalling in beans

In order to demonstrate the possible role of *PvPHR1* in the regulation of gene expression in P-starved bean roots, we undertook the RNAi gene-silencing approach in composite plants. The protocol for the generation of composite bean plants was recently reported as an alternative for stable transformation, especially for species recalcitrant to transformation such as common bean (Estrada-Navarrete *et al.* 2007).

The pTDT-DC-RNAi, with the Gateway cassette driven by the CaMV 35S promoter and the tdTomato (red fluorescent protein) reporter gene (Shaner *et al.* 2004) for identification of the transgenic roots by confocal microscopy, was constructed. This vector was used for the *PvPHR1*-RNAi gene construct consisting of inverted repeats of a *PvPHR1*

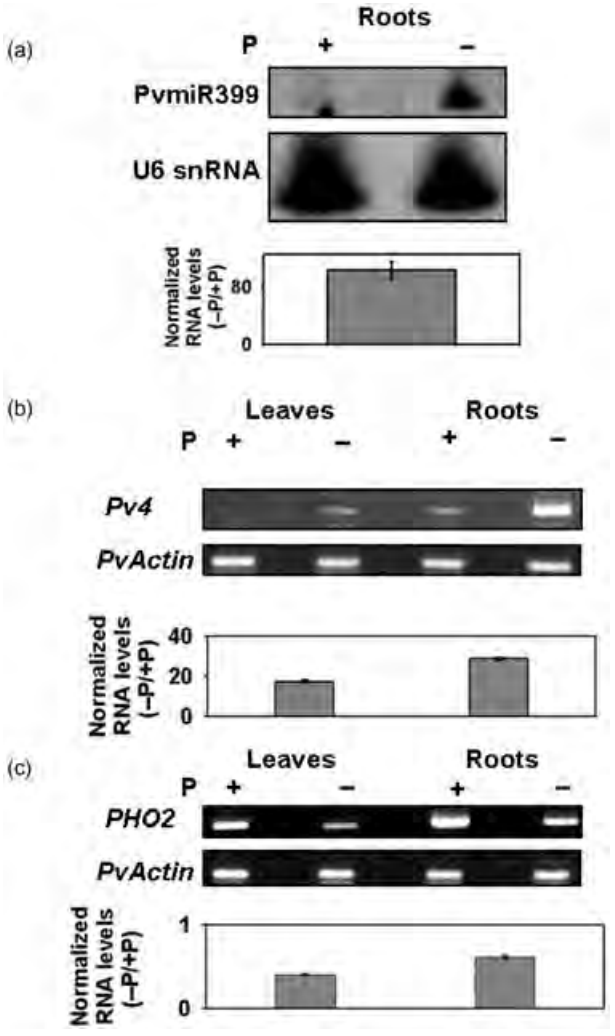

Figure 2. Expression of PvmiR399, *Pv4* and *PvPHO2* in leaves and roots of P-deficient and P-sufficient bean plants. (a) PvmiR399 levels in roots were detected by RNA-blot analysis using U6snRNA as loading control. Transcript levels of *Pv4* (b) and *PvPHO2* (c) in leaves and roots were detected by semiquantitative RT-PCR (sRT-PCR) using the actin gene as control for uniform sRT-PCR conditions. The intensity of the bands was quantified densitometrically, and the –P/+P normalized expression ratios were obtained. Values are mean ± SE of three biological replicates.

gene fragment (Supporting Information Fig. S1a). The expression of *PvPHR1*-RNAi was verified in putative transgenic roots from composite bean plants (Supporting Information Fig. S1b).

In order to confirm *PvPHR1*gene silencing, sRT-PCR analysis was performed in composite bean plants expressing the *PvPHR1*-RNAi construct, as compared with control composite plants transformed with an empty vector. Each transgenic root analysed showed a specific level of *PvPHR1* transcript, indicating different degrees of gene silencing, resulting from a different transformation event in each root. The comparison of *PvPHR1* transcript levels of RNAi versus the control plants grown in –P revealed a reduction ranging from 15 to 95% among the analysed composite plants (Fig. 3a). The level of *PvPHR1* gene silencing showed an inverse correlation with the transcript level of two P-responsive target genes *PvPHT1* (phosphate transporter) and *PvAP5* (Fig. 3a).

To identify the PvPHR1 target genes, we evaluated the transcript level of selected P-deficiency-induced genes (Hernández *et al.* 2007) (Supporting Information Table S1) in the *PvPHR1*-RNAi plant with the highest degree of gene silencing (95%, Fig. 3a) as compared with control (empty vector) composite plants under +P and –P (Fig. 4a). While the –P control plant showed an evident induction of P-responsive genes, the silenced plant showed a reduction of target gene transcript levels (Fig. 4a). Transcript levels of three genes involved in P remobilization, namely *PvAP*, *PvAPC5* and *PvRNS* (RNase), showed a

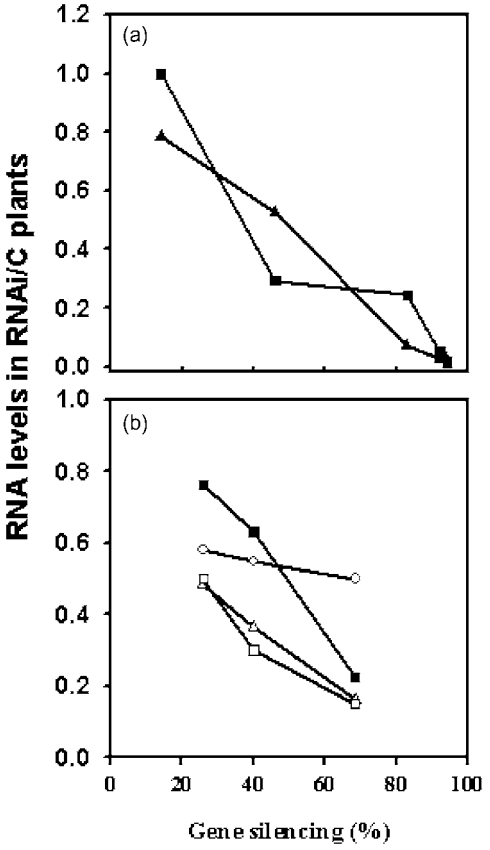

Figure 3. Effect of *PvPHR1* or *PvDCL1* RNAi gene silencing on the expression of P-responsive genes and miRNAs. Data from five *PvPHR1*-RNAi plants (a) and three *PvDCL1*-RNAi plants (b) with increasing percentage of gene silencing each, grown in –P. Transcript levels of *PvPHT1* (■) and *PvAPC5* (▲) were evaluated by semiquantitative RT-PCR (sRT-PCR) in transgenic roots of each composite plant. Transcript levels from PvmiR399 (△) and from orthologs of AtmiR159 (□) and AtmiR160 (○) were evaluated by RNA-blot analysis. The intensity of the bands from amplified products and from hybridization was quantified densitometrically, normalizing that from RNAi silenced plants to that from control (empty vector) plants.

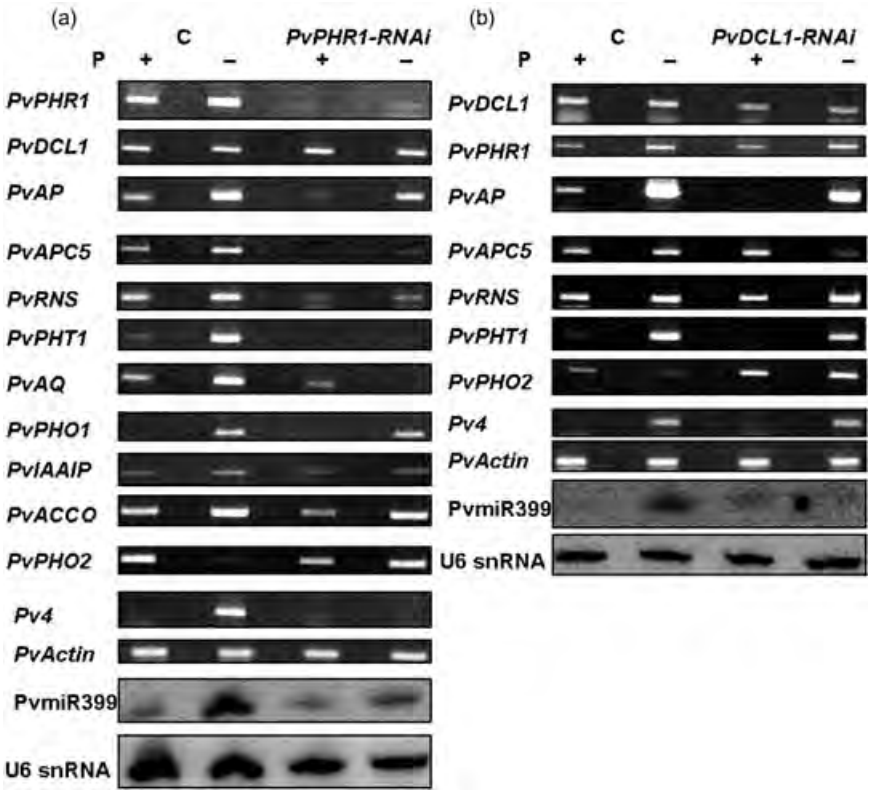

Figure 4. Evaluation of target gene transcript levels in roots of composite bean plants with the highest degree of *PvPHR1* (a) or *PvDCL1* (b) gene silencing. Control (empty vector, C) and RNAi silenced plants grown in +P or –P were analysed. Transcript levels for the indicated *Phaseolus vulgaris* genes were determined by semiquantitative RT-PCR (sRT-PCR), and PvmiR399 levels by RNA-blot analysis using the actin and U6 snRNA as controls, respectively.

reduced response to P deficiency in the *PvPHR1* silenced plant as compared with the control plant. In addition, the –P induction of *PvPHT1* and aquaporin (*PvAQ*), both involved in transport, decreased significantly. The expression of the *PvPHO1* gene, a P translocator involved in loading root P into the xylem vessel, was not affected in *PvPHR1*-RNAi plants. A highly increased expression of *PvPHO2*, involved in P homeostasis, was observed in the *PvPHR1* silenced plant under P deficiency. In addition, the expression of PvmiR399 in –P was significantly reduced despite the unaffected expression of *PvDCL1*. The *Pv4* transcript level was not detected in +P conditions, while in –P, the silenced plant showed a complete inhibition as opposed to the control plant. We determined the transcript levels of 1-aminocyclopropane-1-carboxylate oxidase (*PvACCO*) for ethylene biosynthesis and an indole acetic acid-induced protein gene (*PvIAAIP*). Because both phytohormone-related genes are induced in P-deficient bean roots (Hernández *et al.* 2007), they could be implicated in the root architecture modification response under P starvation (Bonser, Lynch & Snapp 1996; Lynch & Brown 2001; López-Bucio *et al.* 2003). As shown in Fig. 4a, the –P response of these genes was not affected in the *PvPHR1*-RNAi composite bean plants.

The P-deficiency phenotype of bean composite plants was also assessed in relation to the concentration of free P in different plant organs. *PvPHR1*-RNAi composite bean plants showed a decrease of free P content in their leaves (2.5-fold), stems (11-fold) and roots (1.3-fold) as compared with control composite plants (Table 1).

PvmiR399 and PvPHO2 are components of the PvPHR1 signalling pathway in beans

With the rational that miRNA levels would be suppressed in plants with reduced levels of DCL1, we analysed *PvDCL1* silenced composite plants as a tool to investigate the participation of PvmiR399 in the PvPHR1 signalling pathway through the regulation of *PvPHO2*. Composite plants bearing the *PvDCL1*-RNAi gene construct driven by the CaMV 35S promoter (Supporting Information Fig. S1c,d) showed a 20–70% reduction of *PvDCL1* transcript levels as compared with the control plants (Fig. 3b). *PvDCL1*-RNAi composite plants showed an inverse correlation between *PvDCL1* silencing levels and the abundance of three bean miRNAs, homologous to *Arabidopsis* miR399, miR159 and miR160, thus indicating that reduced amounts of *PvDCL1* transcript affected miRNA biogenesis and accumulation (Fig. 3b). An inverse correlation between *PvDCL1* transcript and the P-responsive gene *PvPHT1* was also observed in *PvDCL1*-RNAi plants grown in –P, suggesting the participation of miR399 in signalling for P-deficient response (Fig. 3b).

The *PvDCL1*-RNAi composite plant with the highest degree of gene silencing (70%, Fig. 3b) was further analysed to evaluate the transcript levels of selected P-responsive genes (Hernández *et al.* 2007; Supporting Information Table S1) as compared with control (empty vector) composite plants under +P and –P (Fig. 4b). The *PvDCL1*-RNAi plant showed a significant reduction in the –P response of *PvAP*, *PvAPC5* and *PvPHT1* genes. In

contrast, a high increase in *PvPHO2* transcript levels was observed in the *PvDCL1* silenced plant grown in –P. This behaviour was independent of *PvPHR1*, because the *PvPHR1* –P induction was not affected by *PvDCL1* silencing. Also, the –P gene expression response of *PvRNS* and *Pv4*, two PvPHR1 target genes, was not affected in the *PvDCL1* silenced plant (Fig. 4b).

Although these results suggest the participation of miR399 in the PvPHR1 signalling pathway, the effect of silencing *PvDCL1* may not be solely mediated through this miRNA. *PvDCL1*-RNAi plants showed reduced accumulation of several miRNAs (Fig. 3b) probably involved in diverse biological processes. The effect of other miRNAs on gene expression of –P-responsive genes in bean roots cannot be excluded.

DISCUSSION

In contrast to *Arabidopsis*, common bean and other legumes are not easily amenable to stable genetic transformation, and hence, protocols for high-throughput generation of transgenic legume plants are not available. On the other hand, alternatively, fast, reproducible and efficient protocols for the generation of composite plants, with untransformed shoots using *A. rhizogenes*-mediated transformation, have been established for several legume species including common bean (Estrada-Navarrete *et al.* 2007). This approach had not been used in common bean. In this work, we used the composite plants approach in conjunction with RNAi-silencing technology and showed its feasibility to carry on functional genomics in common bean.

A model summarizing the crucial molecular events in the P-deficiency signalling controlled by *PvPHR1* in bean roots is presented (Fig. 5). Once P deficiency is sensed – either locally or systemically – by unknown molecule(s), this TF positively regulates the expression of target genes involved in P transport (*PvPHT1* and *PvAQ*), remobilization (*PvAP*, *PvAPC5* and *PvRNS*) and homeostasis (*Pv4* and PvmiR399). Transcript analysis of *PvDCL1* silenced plant suggests that PvmiR399 is a negative regulator of *PvPHO2* that can, in turn, directly or indirectly, regulate the expression of P-responsive genes like *PvAPC5*, *PvPHT1* and *PvAP*. However, these effects may also be mediated by other miRNAs that are reduced in PvDCL1 silenced plants. The action of PvmiR399 is apparently regulated by *Pv4* probably through the mimicry mechanism that regulates P homeostasis (Franco-Zorrilla *et al.* 2007). The bean *At4* ortholog that we identified (*Pv4*, TC7206) has a miR399 target site with the same characteristics of *At4*, showing base pairing and a mismatched loop with the PvmiR399 sequence required for the mimicry mechanism.

Silenced *PvPHR1* transgenic roots showed a 23% reduction on free P content under P deficiency as compared with control roots. This phenotype could be related to the drastic reduction of the high-affinity P transporter (*PvPTH1*) that should impair P uptake in the *PHR1*-RNAi plant during P starvation. In addition, a reduction of free P content was observed in non-transformed stems (90%) and leaves (38%) of the silenced plant as compared with plant organs of control composite plants, which can be related to the drastic reduction of *Pv4* transcript, implicated in P homeostasis. *Arabidopsis At4* loss-of-function mutants are impaired in P distribution between roots and shoots during P starvation (Shin *et al.* 2006).

Under P deficiency, bean and other plants modify their root architecture by decreasing primary root growth and increasing lateral roots and root hair formation, and by modifying their root gravitropism to develop shallower and adventitious roots (Bonser *et al.* 1996; Lynch & Brown 2001; López-Bucio *et al.* 2003). However, one of the caveats of the transgenic roots system is its limitation for morphological/physiological analyses of root phenotypes such as the one expected for –P bean plants. We observed that –P induction of the phytohormone-related genes *PvACCO* and *PvIAAIP* was not altered in *PHR1*-RNAi silenced plant. These genes could be involved in modification of root architecture, known to be regulated through phytohormone gradients, principally involving auxins, cytokinins and ethylene (Nacry *et al.* 2005). In *Arabidopsis*, AtWRKY75, AtZAT6 and AtBHLH32, but not AtPHR1, have been implicated in the regulation of root architecture modification (Chen *et al.* 2007; Devaiah *et al.* 2007a,b).

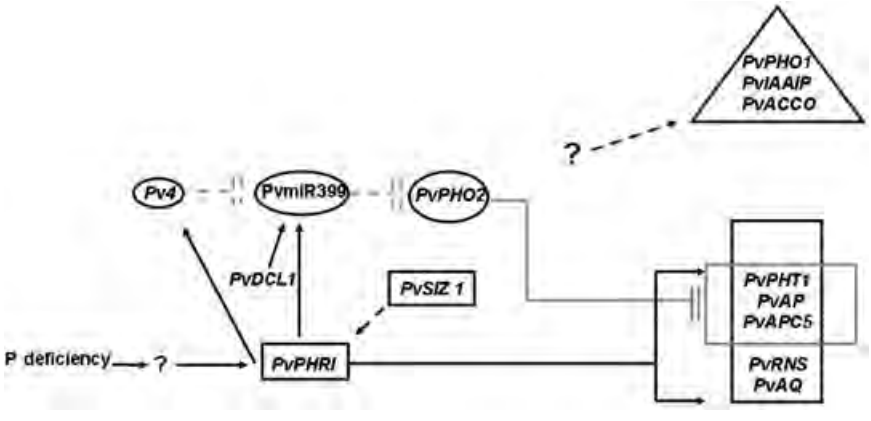

Figure 5. Model for the PHR1 signalling pathway in bean. Positive regulators and the *PvPHR1* target genes are inside the squares. Negative regulators are inside the ovals. The PvmiR399 or *PvPHO2* target genes are inside a grey square. Target genes for an unknown transcription factor (TF) are inside a triangle. Positive regulation is represented with arrows, and negative regulation with grey lines. The dotted lines represent putative positive or negative regulation. Question marks represent unknown receptors or regulators.

The regulation of common target genes by different TF, such as AtPHR1, AtWRKY75 and AtZAT6 (Rubio *et al.* 2001; Devaiah *et al.* 2007a,b), indicates that there is a complex crosstalk occurring during signalling in P-deficient response in *Arabidopsis*. We identified TF candidate genes from different families proposed to be involved in the regulation of P-deficient responses (Graham *et al.* 2006; Hernández *et al.* 2007). Tesfaye *et al.* (2007) have reported that members of the WRKY gene family are P responsive in bean, either by induction or by repression. It is possible that crosstalk among different signalling pathways during P starvation functions in bean, but this remains to be demonstrated.

Recent reviews have emphasized the essential role of TF and miRNAs in gene regulation, representing the largest families of gene regulatory molecules in multicellular organisms. Most of the genes in the eukaryotic genomes are controlled by a combination of transacting factors; miRNAs and TF are linked to one another in gene regulatory networks (Hobert 2008). In this work, we demonstrate the role of a TF, together with a miRNA, in the regulation of P-responsive genes in common bean. It has been proposed that miRNAs are involved in rapid, adaptive changes in gene expression to maintain homeostasis and respond to specific environmental signals (Hobert 2008). In this regard, the role of PvmiR399 could be essential for the regulation of P homeostasis in beans, and its transport into phloem sap, demonstrated in *Brassica napus*, *Cucurbita maxima* and *Oryza sativa* (Buhtz *et al.* 2008; Pant *et al.* 2008; Zhou *et al.* 2008), could be necessary for regulating P homeostasis in different plant organs.

The results presented here set the basis for understanding the signalling events that occur in response to P deficiency in bean, and can be used for improving bean germplasm for tolerance to abiotic stress, either by breeding or by biotechnological approaches.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Diagrams of RNAi constructs and their expression in transgenic roots. Diagrams (not drawn to scale) representing the *PvPHR1*-RNAi (a) and *PvDCL1*-RNAi (c) gene construct Gateway cassettes, cloned in the pTDT-DC-RNAi vector. The constructs consist of inverted repeats of a *PvPHR1* or *PvDCL1* gene fragments, separated by the WRKY intron and driven by the CaMV 35S promoter. Arrows indicate the position of the primers used to verify the orientation of the inverted repeats by PCR amplification. Expression of *PvPHR1*-RNAi (b) and *PvDCL1*-RNAi (d) were determined by sRT-PCR from putative transgenic roots as compared with control (empty vector) roots from composite plants grown in +P and –P conditions. The *TDT* and actin genes were used as internal controls.

Figure S2. Nucleotide sequence of PvmiR399 and *Pv4* and deduced amino acid sequences of PvDCL1 and PvPHO2 and as compared with *Arabidopsis* orthologous genes. (a) Comparison between members of AtmiR399 gene family and homologous PvmiR399 nucleotide sequences. Conserved bases are highlighted in black. (b) Comparison of deduced amino acid sequence of AtDCL1 and PvDCL1; conserved regions corresponding to double-strand RNA-binding domain are highlighted. (c) Alignment of AtmiR399 and PvmiR399 with complementary sequences from *At4* and *Pv4*, respectively. Mispaired nucleotides are shown in red. (d) Comparison of deduced amino acid

sequence of AtPHO2 and PvPHO2; conserved ubiquitin-conjugating enzyme E2 catalytic domain (UBCc) is highlighted.

Table S1. Selected P-responsive common bean genes: annotation, designed primers and sRT-PCR conditions used for expression analysis.

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Title: MicroRNAs expression profile in *Phaseolus vulgaris* bean during nutrient deficiency stresses and manganese toxicity.

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[†] *In memoriam*

Running title: *Nutritional stress-responsive miRNAs in Phaseolus bean*

Total word count: 4 787. **Introduction word count:** 675. **Material and Methods word count:** 1 164. **Results word count:** 1 320. **Discussion word count:** 1 494 **Conclusion word count:** 44. **Acknowledgments word count:** 89
Figures: 6. **Tables:** 0.

Summary (Word count: 200)

- MicroRNAs (miRNAs) are key regulators for Arabidopsis development and stress responses. A hybridization approach using miRNAs-macroarrays was used to identify miRNAs that respond to nutritional stress in *Phaseolus vulgaris*.
- miRNAs-macroarrays were prepared by printing nylon filters with DNA synthetic oligonucleotides (70) complementary to reported conserved and novel (soybean and *P. vulgaris*) miRNAs. The expression ratios of responsive microRNAs were calculated for leaves, roots, and nodules from control or nutrient-stressed (phosphorus, nitrogen, or iron deficiency; acidic pH; and manganese toxicity) *P. vulgaris* bean plants.
- From 32 different miRNAs expressed in leaves, roots, and nodules, 25 showed significant differential expression among the organs tested. Under stress conditions, 35 miRNAs were differentially expressed, 16 of these were responsive to all the stress conditions tested. Importantly, miRNA-blot expression analysis confirmed the macroarrays results. The expression of selected miRNA target genes was evaluated by RT-PCR.
- These results indicate that miRNAs, such as miR156/miR157, miR165/miR166, and gma-miR1532, may have relevant roles in *P. vulgaris* root and nodule development, while some of these have only been reported for Arabidopsis leaf and flower development. MiR167 and miR170, previously known to be involved in developmental processes, may also participate in *P. vulgaris* nutritional stress responses.

Key words: *Phaseolus vulgaris* (common bean), nutrient-deficiency stress, manganese toxicity, *P. vulgaris* microRNAs, nutrient-stress responsive microRNAs.

Introduction

Like other eukaryotes, plants need to regulate the expression of hundreds or thousands of genes to ensure proper development, tissue function, and adequate response to environmental changes. Regulation of gene expression is complex; it can be regulated at the transcriptional, posttranscriptional, or translational level. Regulatory mechanisms require the participation of different transcription factors (TFs) and non-protein coding RNAs (npcRNA) (Hobert, 2008; Jones-Rhoades *et al*., 2006; Voinnet, 2009). Recent studies have emphasized the synergistic role of TFs and npcRNA in regulating gene expression. Likewise, a putative role for npcRNAs as mobile signal molecules has been proposed in plants (Buhtz *et al*., 2008; Hobert, 2008; Kawashima *et. al*., 2009; Pant *et al*., 2008)

npcRNAs are a class of riboregulators of variable length (18 to 1,000 nucleotides). MicroRNAs (18-24 nt), the most studied npcRNAs family, regulate gene expression through target gene transcript cleavage, translational inhibition, or chromatin modification (Jones-Rhoades *et al*., 2006; Ben Amor *et al*., 2009; Voinnet, 2009). In plants, some 29 microRNA (miRNA) families are conserved; however, recent reports indicate that miRNAs can be organ-, species-, physiological event-, or stress-specific. For example, specific miRNAs have been reported for soybean nodule development and maintenance, *P. vulgaris* drought responses, tomato fruit development, nutritional stress and heavy metal toxicity (Moxon *et al*., 2008; Subramanian *et al*., 2008; Liu *et al*., 2008; Arenas-Huertero *et al*., 2009; Huang *et al*., 2009). Additionally, strong evidence exists for the participation of miRNAs in regulating different developmental and physiological processes, such as leaf and nodule development, auxin signaling, phase transition, flowering, as well as response to abiotic stress and pathogen attack (Combier *et al*., 2006; Mallory & Vaucheret, 2006; Navarro *et al*., 2006; Chuck *et al*., 2008). miRNAs also participate in phosphorus (P), copper (Cu), and sulfur (S) homeostasis; in these cases miR399, miR398/miR408, and miR395 are induced and in part regulate the gene expression of *UBC-24* (*PHO2*), *Cu/Zn superoxide dismutase* (*CSD1 and CSD2*), and *ATP sulphurylase* (*APS4*) and *SULTR*, respectively (Abdel-Ghany & Pilon 2008; Chiou *et al*., 2006; Kawashima *et al*., 2009; Li *et al*., 2008). Likewise, miRNA cloning from and expression in cold, drought, UV, salinity, flooding, and heavy metal stressed plants has been reported (B. Zhao *et al*., 2007; Zhang *et al*., 2008; X. Zhou *et al*., 2008; Arenas-Huertero *et al*., 2009; Huang *et al*., 2009; Zhao *et al*., 2009)

Phaseolus vulgaris (*P. vulgaris*, common bean) is the most important grain legume for direct human consumption. Environmental factors such as low soil nitrogen (-N) and phosphate (-P) levels, and acid soil conditions (low pH) are important constraints for *P. vulgaris* production in most areas of Latin America and Africa where the crop is grown (Broughton *et al*., 2003; Graham *et al*., 2003). *P. vulgaris* has evolved morphological, physiological, and biochemical adaptive responses to cope with these constraints. Such responses include root architecture modification, organic acid exudation, anthocyanin accumulation, activation of high affinity transporters for nutrients, and symbiotic interactions (Raghothama 1999; Smith 2001; Vance *et al*., 2003; Valdés-López & Hernández, 2008). There is a paucity of information on the regulation of *P. vulgaris* responses to stress. Although it has been demonstrated that miR399 is a component of the P-deficiency signaling pathway in *P. vulgaris* (Valdés-López et al. 2008) and that several *P. vulgaris* miRNAs have been recently reported (Arenas-Huertero *et al*., 2009), information is lacking about the putative participation of miRNAs in regulating nutrient-deficiency-responsive genes.

The aim of this work was to identify nutrient-stress responsive miRNAs in leaves, roots, and nodules from *P. vulgaris* plants subjected to P, N, or iron (Fe) deficiency, as well as acid pH (5.5) and manganese toxicity (++Mn). Hybridization of macroarrays printed with synthetic DNA oligonucleotides complementary to conserved miRNAs and novel miRNAs from *P. vulgaris* and soybean were used to identify miRNAs that showed differential expression under nutritional-stress conditions. We determined that some miRNA have organ-specific expression patterns in bean, even under the same stress conditions. We propose that nutritional-stress-responsive miRNAs and their target gene transcripts are involved in the regulating the signaling pathways for the adaptation of *P. vulgaris* plants to different stress conditions.

Materials and Methods

Plant materials and growth conditions

The *P. vulgaris* Mesoamerican 'Negro Jamapa 81' cultivar was used in this study. Seeds were surface sterilized and germinated on sterile and moist verculite at 25 °C for 3 days. Seedlings were transplanted to plastic boxes containing 8 L of Franco/Munns nutrient solution (Franco & Munns, 1982). The nutrient solution was aerated with aquarium air pumps and the volume and pH (6.5) were adjusted daily. Plants were grown in this hydroponic system in a greenhouse with controlled environment conditions (25-27 °C, 70% humidity, and natural illumination). After 7 days, the nutrient solution was changed and nutritional stresses were established. One set of these plants was grown under optimal (control) nutritional conditions. To induce the P or Fe deficiencies, the concentration of each element was diminished to 2 μM, whereas in the control solution these nutrients were was 200 (P) and 100 (Fe) μM. For N deficiency, the solution was depleted of any N source. For ++Mn, the nutrient solution was supplemented with 200 μM $MnCl_2$. For acid conditions, pH was adjusted to 5.5. Both controls and treatments consisted of 3 independent plastic boxes, 8 seedlings per box, 24 plants total. After 7 days of treatment, leaves and roots were harvested and immediately collected in liquid N_2 and stored at -80 °C until used for RNA isolation. To evaluate the miRNA expression in nodules, the same hydroponic system with N-free nutrient solution and a bacterial inoculum of *Rhizobium tropici* CIAT 899 was used. Each box for plant growth contained 10^7-10^8 bacterial cells (Franco & Munns, 1982). After 10-12 days post-inoculation (dpi), when plants had functional nodules, the same nutrient-stress treatments described previously were initiated. After 7 days of treatment, nodules were collected in liquid N_2 and stored at -80 °C until analyzed.

Inductively coupled plasma mass spectroscopy (ICP-MS) analysis and nitrogenase activity

The ICP-MS analysis for shoots and roots from non-nodulated plants was done as reported by Jain *et al*. (2009). Nitrogenase activity was determined in detached, 21 dpi nodulated roots by the acetylene reduction assay (ARA) as reported by Mendoza *et al.* (1995).

miRNA purification

Total RNA was isolated from 1g frozen leaves, roots, or nodules from control or –P, -Fe, -N, pH 5.5, or ++Mn *P. vulgaris* plants as reported by (Valdés-López *et al.* (2008). For miRNAs isolation, 100 μg of total RNA was fractionated and cleaned using the flashPAGE fractionator and flashPAGE Reaction Clean-up (Ambion, Austin TX, USA), respectively. The miRNA samples were stored at -80 °C until tested.

Preparation and hybridization of miRNA-macroarrays

Seventy synthetic DNA oligonucleotides (18-24 nts) corresponding to reverse complementary sequences of different mature miRNAs were synthesized. (Sequences of each oligonucleotide are provided in Supporting Information Table S1.) Additionally, 2 oligonucleotides, corresponding to T7 and M13 bacteriophage promoters, were used as negative controls since these did not show complementarity to any known miRNAs. Twenty-four of these DNA oligonucleotides represent an equal number of members of different conserved miRNA families in several plant species (Sunkar & Jagadeeswaran, 2008). The other DNA oligonucleotides correspond to soybean (35) and *P. vulgaris* (11) novel miRNAs that were cloned from bean seedlings or from soybean roots inoculated with *Bradyrhizobium japonicum*, respectively (Subramanian *et al*, 2008; Arenas-Huertero *et al*, 2009). Each probe was manually spotted on 2 x 3 cm Amersham Hybond-N+ membranes (GE Healthcare, Buckinghamshire, UK), dried at room temperature, and UV-fixed three times. Printed membranes (miRNAs-macroarrays) were wrapped in aluminum foil and stored at -20 °C until evaluated.

For miRNA-macroarray hybridization, isolated miRNAs from nutrient and pH stressed or control *P. vulgaris* organs were dephosphorylated with Antarctic Phosphatase (New England Biolabs, Beverly, MA, USA) and then radioactively labeled with T4 Polynucleotide kinase (PNK) (New England Biolabs, Beverly, MA, USA) and [γ-^{32}P]-ATP (Perkin-Elmer, Boston, MA, USA). The labeling reaction was performed at 37 °C for 1 hr and stopped by incubation at 90 °C for 5 min and then the probe was incubated in ice for 3 min. The miRNA-macroarrays were pre-hybridized in 1 ml of ULTRAhyb-Oligo Hybridization Buffer (Ambion, Austin TX, USA) at 37 °C for 1 hr. The probe was added to hybridization solution for 15 hrs at 37 °C. The hybridized macroarrays were washed twice in 2X SSC/0.1X SDS for 15 min at 37 °C and then washed 3 to 5 times more with the same washing solution and temperature for 6 min each time. The membranes were then exposed to a Phosphor Screen System (GE Healthcare, Buckinghamshire, UK) for 1 day. The phosphor screen was scanned in a Storm 860 Gel and Blot Imaging System (GE Healthcare, Buckinghamshire, UK). Three independent macroarrays were hybridized with miRNAs isolated from three different biological samples of each treatment and organ. The fluorescence intensity of each spot was quantified using the ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA, USA). Linear regression analysis was performed on each replicate of each treatment; only those replicates for which the linear regression could explain at least 80% of the variation ($r^2 \geq 0.8$) were considered. For analysis, the data were adjusted with the average of the signal intensity of the printed miRNA: pvu-miR482*, which showed no significant variation across conditions tested as well as in other abiotic stresses (Arenas-Huertero *et al.*, 2009). The normalized data were then used to obtain the expression ratios: treatment/control for nutritional-stress responsive miRNAs, or organ/organ for control growth conditions. Student's *t*-test was performed with a *P*-value cutoff of ≤ 0.05. Only 1.5 or higher with ratios of $P \leq 0.05$ were considered. To identify organ-specific miRNAs, as well as stress-specific miRNAs and/or common

miRNAs between stresses, Venn diagram and Flower diagram analyses were performed.

miRNA-blot Analysis

For miRNA-blot analysis, total RNA was isolated as reported (Valdés-López *et al.*, 2008), and 20 μg of total RNA was separated by 15% PAGE/8 M urea/1 X TBE Buffer. The gel was electroblotted to Hybond-N^+ membrane (GE Healthcare) and then UV cross-linked twice. Hybridizations were performed at 42 °C during 15 hrs in UltraHyb-oligo solution (Ambion, Austin, TX, USA). Hybridized membranes were washed twice in 2x SSC/0.1% SDS for 30 minutes each time, and then exposed to Phosphor Screen System (GE Healthcare, UK). The fluorescence intensity was quantified as mentioned above. Synthetic DNA oligonucleotides with the antisense sequence corresponding to miRNAs (Supporting Information Table S1) were used as probes after 5'-end-labeled using [γ-^{32}P]-ATP (Perkin-Elmer, Boston, MA) and T4 PNK (New England Biolabs, Beverly, MA, USA). The probes were purified with Quick spin oligo columns (Roche, Indianapolis, IN) before addition to hybridization solution. As control, loading a DNA oligonucleotide complementary to U6 snRNA was used as probe.

Target validation

cDNA was synthesized from 5 μg total RNA using an oligo-dT primer. The amount of cDNA was standardized to the abundance of *actin* gene (Valdés-López *et al*., 2008). Semiquantitative RT-PCR (sRT-PCR) analysis was performed to assess the correlation between miR156/miR157 and miR170 expression and each target gene transcript expression. Target gene sequences were obtained from Arenas-Huertero *et al*., 2009. PCR condition and primers sequences are described in the Supporting Information Table S2.

Results

Phenotypic characterization

To analyze the miRNA expression profile in *P. vulgaris* due to different nutritional stresses, plants were grown in hydroponic conditions with –P, -N, -Fe, pH 5.5, or ++Mn for 7 days. Except for –N, these treatments were also applied to nodulated *P. vulgaris* plants. Compared with control plants, a reduction between 3-4 fold in N, P, and Fe content was observed in –N, -P, and –Fe-plants, respectively (Fig. 1a-c), whereas a striking increase in Mn content was recorded in ++Mn-plants (Fig. 1d). For pH 5.5 plants, a 2-fold decrease was observed in Mn, Cu, and Ca (Fig 1d-e). Plants grown in each stress condition showed characteristic visible symptoms. N-deficient plants had chlorotic and purple leaves; -Fe plants showed chlorotic leaves, and ++Mn plants had leaves with brown spots (Supporting Information Fig. S1). In addition, –P, -N, and pH 5.5 plants showed a decrease in the dry weight, whereas –Fe and ++Mn plants were not affected in this parameter (Fig. 2a). Even though the nodulated plants received the same stress as non-nodulated plants, these plants were not affected in biomass production (Fig. 2b). However, stressed nodulated plants showed reduced nodule dry weight and nitrogenase activity when compared to control plants (Fig. 2c-d). Because these results showed that each applied treatment induced the expected stress

phenotype, the plants from these experimental systems were used to analyze responses regarding differential miRNA expression.

MiRNAs expressed under optimal nutrient conditions in different *P. vulgaris* plant organs.

The participation of miRNAs in plant development has been demonstrated in many different plant species (Chuck *et al*., 2008; Gregory *et al.*, 2008). Although the expression of conserved and novel bean miRNAs was reported recently (Arenas-Huertero *et al*., 2009), little information is available about the expression of bean miRNAs and novel soybean miRNAs in different *P. vulgaris* organs, including nodules. To address this question, hybridization of miRNA-macroarrays was performed to analyze the miRNAs expression profile of leaves, roots, and nodules of *P. vulgaris* plants grown under optimal nutritional conditions. From the 70 miRNAs printed on the macroarrays, expression for only 32 miRNAs was detected in the organs analyzed from control plants (Supporting Information Table S3). Nineteen of these miRNAs were expressed in all three analyzed organs. Three miRNAs (miR398, miR408, and gma-miR1508) were expressed only in leaves and roots while two miRNAs (miR167 and pvu-miR1511) were common between leaves and nodules. Additionally, seven miRNAs (miR169, miR319, miR393, miR397, miR399, pvu-miR1509, pvu-miR1510) were only expressed in leaves, one miRNA (miR172) was nodule specific (Fig. 3a). These data suggest that the 19 miRNAs expressed in all 3 organs may be relevant for organ development or functional maintenance; while the leaves and nodule-specific miRNAs may be required to maintain a specific organ function.

Even though several miRNAs were expressed in all three organs, their relative accumulation in each organ was different (Fig. 3b). From 32 expressed miRNAs in our analysis, 25 of them showed significant ($P \leq 0.05$) differential accumulation between these organs (Fig. 3b). Examples of these were miR396, pvu-miR2118, pvu-miRS1, and pvu-miR1511, as well as pvu-miR2119, pvu-miR1509, and gma-miR1526, preferentially accumulated in roots and leaves, respectively. For nodules, only miR172 and pvu-miR1511 showed a preferential induction in these organs. Our findings suggest that these miRNAs may have relevance in leaf, root, and nodule development; however, further experimentation will be required to identify any functional role in these organs.

MiRNAs that participate in development processes and other abiotic stress responses in Arabidopsis were expressed in nutrient-deficient and ++Mn bean plants

The participation of miRNAs in biotic and abiotic stresses, as well as in the P-, Cu-, and S-homeostasis has been demonstrated for Arabidopsis (Bari *et al*., 2006; Yamasaki *et al*., 2008; Kawashima *et al*., 2009). Despite the agronomic importance of *P. vulgaris*, information is limited about the expression of miRNAs during adaptation to nutritional stresses and heavy metal contamination. To address this limitation we evaluated the expression of miRNAs in *P. vulgaris* plants under -P, -N, -Fe, pH 5.5, or ++Mn stresses. We found that 35 miRNAs responded to the different treatment conditions, showing different expression patterns in leaves, roots, and nodules (Fig. 4). Among the responsive miRNAs, miR156/miR157, miR167, miR319, miR396, pvu-miR1509, pvu-miR1511, gma-1524, gma-miR1526, and gma-miR1532 displayed general up-regulation in response to all the stress treatments and organs analyzed (Fig 4a). Other miRNAs (miR169, miR397, miR408, and pvu-miR1510) generally displayed down-regulation in all the experimental conditions. Also, miRNAs that were

preferentially induced in a specific tissue were detected. For example pvu-miR2118 was induced in nodules but was repressed in leaves (Fig. 4a). We analyzed the miRNA-macroarray experiments data through flower diagram analysis. This analysis led us to identify 16 miRNAs that responded to –P, -Fe, pH 5.5, and ++Mn; that herein will be referred to as common stress-miRNAs (Fig. 4b and Supporting Information Table S5). Only four of the common stress miRNAs have been implicated in other abiotic stresses, such as Cu deficiency in Arabidopsis (miR398, miR408) and in drought and salinity in bean (pvu-miR2118 and pvu-miR159.2) (Yamasaki *et al*., 2008; Arenas-Huertero *et al*. 2009). The expression of some miRNAs was common for two or three different stresses: five miRNAs (miR164, miR395, pvu-miR1509, pvu-miR1510, and gma-miR1508) were in common between -Fe, pH 5.5, and ++Mn, and two miRNAs (miR166 and miR172) were in common between -P, -Fe, and ++Mn. In addition, three miRNAs were –Fe specific (pvu-miR1514a, gma-miR1515, and gma-miR1516) and only the miRNA gma-miR1511 was specific for pH 5.5 plants (Fig. 4b). In addition to miR399 (Valdés-López *et al*., 2008), we detected nine miRNAs (miR156/miR157, miR160, miR165/miR166, miR169, miR393, pvu-miR2118, gma-miR1524, gma-miR1526, and gma-miR1532) that were expressed in –P conditions, these miRNAs have not been reported as expressed in other P-stressed plant species. Our data provide additional support that in *P. vulgaris* plants miRNAs may be involved in both plant development and in the nutrient deficient adaptation responses.

Expression of selected miRNAs by RNA-blot analysis

Eight miRNAs, including both up-regulated and down-regulated ones, were selected to confirm their expression ratio as observed in the macroarray experiments by the alternative method of RNA-blot. As shown in Figure 5, the pattern of accumulation determined in the miRNA-macroarray experiments of the selected miRNAs was confirmed by miRNA-blot analysis. The only exception was the expression of the miR157 in leaves (Fig. 5). Even though the macroarrays results were validated in 90% of the cases through RNA-blot, with respect to the levels of induction or repression, the observed difference in expression values might be related to the variation in sensitivity between macroarrays and miRNA blots. However, the confirmation by miRNA-blot indicates that in most instances the miRNA-macroarray approach is useful to explore their expression when comparing different growth conditions, and in particular in the response of *P. vulgaris* to many stress conditions.

Target gene transcript expression showed negative correlation with miRNAs expression

A negative correlation between the expression of miRNAs and their respective target gene transcripts has been observed in many cases (Abdel-Ghany & Pilon 2008). A recent report (Arenas-Huertero *et al*., 2009) identified the target mRNAs for several conserved *P. vulgaris* miRNAs and validated their interaction by 5' RACE. We carried out sRT-PCR analysis in order to determine whether the expression of nutritional-deficiency responsive-miRNAs miR156/miR157 and miR170 (Fig. 5) were negatively correlated with their target mRNAs: (*Squamosa promoter binding*: *SPL6*) and miR170 (*Scarecrow like*: *SCL6*), respectively (Arenas-Huertero *et al*., 2009). Compared with control leaves, we detected an increase of the *SPL6* transcripts in –P, -Fe, and ++Mn-leaves (Fig 6a), which is in agreement with the down-regulation of miR157 expression in these stress conditions (Fig. 5). By contrast, the *SPL6* transcript levels in stressed nodules was quite diminished (Fig. 6b), which agrees with the up-regulation of miR157

in this organ (Fig 5). We found a similar response for *SCL6* and miR170 (Fig. 6c). These results support the interpretation that miR156/miR157 and miR170 participate in regulating the expression of their target genes in *P. vulgaris* plants growing under nutrient deficiency conditions and ++Mn.

Discussion

Participation of miRNAs in development/maintenance of *P. vulgaris* organs

Plants containing mutations in genes encoding proteins involved in miRNA biogenesis or function (AGO1, DCL1, HEN1, HYL1, SERRATE, HST, and DDL) exhibit a myriad of dramatic developmental abnormalities, supporting the importance of miRNAs in growth and differentiation (Boutet *et al*., 2003; Lobbes, *et al.*, 2006; Yang *et al*., 2006; Yu *et al*., 2008; Gregory *et al*., 2008). These studies have been conducted primarily in Arabidopsis but not in other plant species such as legumes. Despite the agronomic importance of legumes and the cloning of several miRNAs in different legumes (Subramanian *et al*., 2008; Zhang *et al*., 2008; Z.S. Zhou *et al*., 2008; Arenas-Huertero *et al*., 2009), studies are limited of miRNA expression in growth and differentiation. Thus far, only the participation of miR166 and miR169 in root and nodule development has been demonstrated in *Medicago truncatula* (Combier *et al*., 2006; Boualem *et al*., 2008). Here we report the expression of 32 miRNAs, including conserved miRNAs and novel *P. vulgaris* and soybean miRNAs (Subramanian *et al*, 2008; Sunkar & Jagadeeswaran, 2008; Arenas-Huertero *et al*., 2009), in healthy *P. vulgaris* leaves, roots, and nodules. Few of these miRNA were organ specific, whereas 19 of them were expressed in common in leaves, roots, and nodules. These miRNAs may be involved in *P. vulgaris* leaf and root development, since the conserved miRNAs detected in this work have been found in Arabidopsis developmental processes such as vegetative phase change, flowering, leaf morphology, root architecture development, and senescence (Llave *et al*., 2002; Wang *et al*., 2005; Mallory & Vaucheret 2006; Q. Liu *et al*., 2009).

Regarding miRNA expression in *P. vulgaris* nodules, we propose that gma-miR1524 and miR172 may be involved in nodule development. Based on sequence homology, we found that gma-miR1524 has a AP2-EREBP TF as putative target mRNA (data not shown), and this TF is a positive regulator of nodulation in *Lotus japonicus* (Asamizu *et al*., 2008). MiR172 was detected only in nodules, which is in agreement with Wang *et al*., 2009, where miR172 was the most abundant miRNA in nitrogen-fixing soybean nodule miRNAs libraries. To date, miR172 has been shown to be involved in the photoperiodic flowering pathway and the floral stem cell fate (Jung *et al*., 2007; L. Zhao *et al*., 2007), but participation of this miRNA in nodule development is not known.

Our data support an interpretation that *P. vulgaris* miRNAs are associated with organ development and function. However, these data raise the following questions: what is the physiological role of miRNAs known to be involved in flowering, leaf polarity, and senescence in *P. vulgaris* roots and nodule development? Are these miRNAs regulating several target gene transcripts in nodules or in stress conditions? It has been suggested that bioinformatic target search algorithms might underestimate the number of *in vivo* targets because most of them are based on often unwarranted assumptions regarding the structure and evolutionary conservation of miRNA target sites. In addition, several algorithms consider a true miRNA target only when the mRNA::miRNA base-pairing does not have any mismatches in the seed region of the

miRNA. But recent reports indicate that miRNAs can regulate targets even with some mismatches in the seed region (Brodersen *et al*., 2008; Brodersen & Voinnet, 2009). In this regard, novel approaches to demonstrate that miRNAs could have more target mRNAs *in vivo* have been developed (Addo-Quaye *et al*., 2008; German *et al*., 2008; Franco-Zorrilla *et al*., 2009). Thus, the expressed miRNAs found in this work may have other as yet unidentified target mRNAs in *P. vulgaris*. We suggest that a specific target mRNA for each known miRNA may be specific to a given physiological or stress condition; however, further experimentation is needed to demonstrate this idea and to describe the physiological role of miRNAs that are possibly involved in the development of each *P. vulgaris* organ.

Conserved and novel miRNAs implicated in the adaptation of *P. vulgaris* to nutrient deficiency stresses and manganese toxicity

Information available about miRNA participation in biotic and abiotic stresses is more limited than that on plant growth and differentiation. Micro-array and deep-sequencing approaches have been used to detect miRNAs implicated in the regulation of cold, drought, salinity, flooding, and Cd toxicity responses (B. Zhao *et al*., 2007; H.H. Liu *et al*., 2008; X. Zhou *et al*., 2008; Huang *et al*; 2009). Using these strategies, miR169 has been shown to be involved in drought and salinity tolerance in Arabidopsis and rice (Li *et al*., 2008; B. Zhao *et al*. 2009), and seven heavy metal responsive-miRNAs have been reported in *Medicago truncatula* (Z.S. Zhou, *et al*., 2008). In our work we reported 35 nutrient-deficient and Mn toxicity responsive miRNAs in *P. vulgaris* leaves, roots, and nodules.

In different plant species, including *P. vulgaris*, miR399 and *ISP1*-gene family members have been implicated in the regulation of P-homeostasis (Bari *et al*., 2006; Franco-Zorrilla *et al*., 2007; Valdés-López *et al*., 2008). Recently, miR144, miR827, and miR778 were identified as P-deficiency responsive miRNAs in Arabidopsis (Pant *et al*., 2009). Also it was suggested that miR144 and miR778 can participate in the regulation of chromatin status and miR827 in the anthocyanin synthesis under P-deficient conditions (Pant *et al*., 2009). Here, we reported nine P-deficiency responsive miRNAs (miR156/miR157, miR160, miR165/miR166, miR169, miR393, pvu-miR2118, gma-miR1524, gma-miR1526, and gma-miR1532) in *P. vulgaris*. Bioinformatic analysis based on the soybean gene-index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=soybean) revealed that gma-miR1524 and gma-miR1532 have complementarity with *AP2-EREBP* and *TIR1/CLAVATA1* genes, respectively (data not shown). The *AP2/EREBP* TF gene is involved in root and nodule development in *Lotus japonicus* plants (Asamizu *et al*., 2008). The *TIR1* gene is involved in auxin sensing in P-stressed Arabidopsis plants, whereas *CLAVATA1* is involved in control of cell proliferation in shoot meristems and in the long distance control of nodulation (Searle *et al*., 2003; Magori *et al*., 2009; Pérez-Torres *et al*., 2009). Thus, we propose that the orthologs of *AP2-EREBP*, *TIR1*, and *CLAVATA1* mRNAs in *P. vulgaris* and their regulation by miRNAs are involved in root/nodule development and auxin signaling.

In addition to low P levels, *P. vulgaris* production is affected by low soil N, Fe, acid soil conditions, and heavy metal toxicity. As in other abiotic stresses, responses to nutritional stresses are regulated at different levels, including transcriptional and post-transcriptional levels. In this regard, the participation of miR167 in the response to N-deficiency has been reported in Arabidopsis (Gifford *et al*., 2008; Pant *et al*., 2009), unfortunately, there are no reports about miRNA expression in –Fe, acid soil condition,

or ++Mn. Here, we reported the identification of 31 –Fe, pH 5.5, and ++Mn-responsive miRNAs. Plants cope with these stresses by root architecture modification, nodule-number regulation, activation of high affinity transporter, and nutrient homeostasis (Valdés-López & Hernández, 2008). Among the miRNAs identified in –Fe, -N, low pH, and ++Mn treated plants, we detected miRNAs that have been reported to be involved in root and nodule development (miR160, miR170, miR172, and gma-miR1524), flowering (miR156/miR157), nutrient homeostasis (miR395, miR398, and miR399), drought tolerance (miR169, pvu-miR2118, and pvumiR159.2) and nitrogen deficiency (miR167) (Mallory & Vaucheret, 2006; Gifford *et al*., 2008; Arenas-Huertero *et al*., 2009). Recently, the cloning of six Cd toxicity responsive miRNAs in rice was reported (Huang *et al*., 2009). Also, the putative participation of miR171, miR319, miR393, miR160, and miR166 in the responses to Hg, Cd, and Al toxicity was reported in *Medicago truncatula* (Z.S. Zhou *et al*., 2008). Here, we reported the induction of miR160, miR166, and miR319 in response to ++Mn. Moreover, we detected that miR156/miR157 was 6.5-fold induced and 2-fold down-regulated in ++Mn-stressed nodules and leaves, respectively. In addition, miRNA160 and miR166 have been involved in root and nodule development (Boualem *et al*., 2008). We propose that under ++Mn, where a decrease in nodule dry weight was observed, miR160 and miR166 could be involved in nodule development, as occurs in *Medicago truncatula*. MiR319 has been associated with the jasmonic acid signaling pathway and senescence (Schommer *et al*., 2008) and some ++Mn responses are linked to jasmonic acid and senescence (Fecht-Christofers *et al*., 2003; Pittman, 2005). The participation of miR319 may be in regulating the expression of target gene transcripts that participate in these pathways. Based on previous reports, we cannot explain the participation of miR156/miR157 in the ++Mn responses in root-nodules, because several reports indicate that this miRNA is involved in the processes of vegetative phase change and flower development (Wu & Poething, 2006; Gandikota *et al*., 2007). Physiological validation of miRNAs in ++Mn-stressed root nodules is needed to understand their role under Mn-toxicity. Lastly, we propose the participation of trans-acting small RNAs (ta-si-RNA) in nutrient-deficiency bean adaptation, because miR390, which participates in the biogenesis of the non-protein coding ta-si-RNAs (Montgomery *et al*., 2008), was nutrient deficiency and ++Mn-responsive. Our results suggest that expression of numerous miRNAs during –N, -Fe, low pH, and ++Mn stress may be important in signaling and development in *P. vulgaris*. The information also provides the foundation to evaluate the individual roles of small RNAs in the post-transcriptional responses to nutritional limitation.

Conclusion

The discovery of novel nutrient-responsive miRNAs in *P. vulgaris* contributes to further understanding in the regulation and signaling of this agronomically important plant to nutritional stresses. Further research based on genetic approaches will be required to demonstrate specific roles of miRNAs in bean.

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This work is a small tribute to Peter Graham, a great man and scientist.

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Figure Legends

Figure 1 Inductively coupled plasma mass spectroscopy (ICP-MS) nutrient analysis of shoots of *P. vulgaris* plants grown under control or stress conditions. (a) Nitrogen, (b) Phosphate, (c) Iron, (d) Manganese, (e) Calcium, and (f) Copper contents. Values are mean ± SE from 2 replicates; each one contains six pooled-plants. Asterisks on the histograms indicate that the means differ significantly: *P=0.05, **P≤0.03, *** P≤0.01.

Figure 2 Physiological responses of *P. vulgaris* to nutrient deficiency stress and Mn toxicity. Dry weight of whole non-nodulated plants (a) and nodulated-plants (b). (c) Nodule dry weight. (d) Nitrogenase Activity. Seven-day-old plants were stressed with –P, -N, -Fe, pH 5.5, and ++Mn for 7 days. Values are mean ± SE from 6 biological replicates. Asterisks on the histograms indicate that the means differ significantly (P≤0.05)

Figure 3 MiRNAs expressed in leaves, roots, and nodules of bean plants grown on sufficient (control) conditions. (a) Venn-diagram analysis. (b) miRNA expression profile of different organs. The color scale indicates the different organ/organ ratio values. Only miRNAs with a differential expression (threshold ≥ 1.5 or ≤ -1.5) and with P≤0.05 were considered for this analysis.

Figure 4 MiRNAs that respond to nutrient deficiency and manganese toxicity in different *P. vulgaris* organs. (a) miRNA expression profiles of different stresses and organs. MiRNAs expressed in all the treatments tested are labeled with an asterisk. (b) Flower diagram analysis; the matrix for each stress condition contains the differentially expressed miRNAs in leaves, roots, and nodules. The color scaling indicates the miRNA expression ratio in each stress treatment as compared to control conditions. Only miRNA expression ratios with a threshold ≥ 1.5 or ≤ -1.5 and with P≤0.05 were considered for this analysis.

Figure 5 Verification of microRNAs-macroarray results by miRNA blot analysis. Seven-day-old plants were exposed to different conditions for 7 days: sufficient nutrient conditions (C), P-deficiency (-P), N-deficiency (-N), Fe-deficiency (-Fe), acidic conditions (pH 5.5), or Mn toxicity (++Mn). Total RNA (20 μg) from control or stressed leaves, roots, or nodules was resolved in a 15% PAGE and used for miRNA-blot analysis using synthetic-DNA oligonucleotide probes for individual miRNAs. Detection of U6 snRNA was used as a loading control. Signal intensity was obtained to determine relative miRNA abundance. The level of the control sample was set at 1.0 and the numbers below each line indicate the corresponding fold-exchange. Each miRNA-blot was repeated three times and a representative gel is shown. Sequences to probe each miRNA are described in the Supporting Information Table S1.

Figure 6 Negative correlation between miRNA expression and its target mRNA level. Seven-day-old plants were exposed to different conditions for 7 days: sufficient nutrient conditions (C), P-deficiency (-P), N-deficiency (-N), Fe-deficiency (-Fe), acidic conditions (pH 5.5), or Mn toxicity (++Mn). Five μg of total RNA, from control or stressed leaves, roots, or nodules, was used to synthesize cDNA. The following mRNA fragments were amplified from cDNA synthesized from each sample: (a) *Squamosa binding promoter like protein-6* (*SPL6*) expression in leaves. (b) *SPL6* expression in nodules, and (c) *Scarecrow-like* (*SCL*) expression in roots. The primer sequences and reaction conditions used are presented in the Supporting Information Table S2.

Figure 1

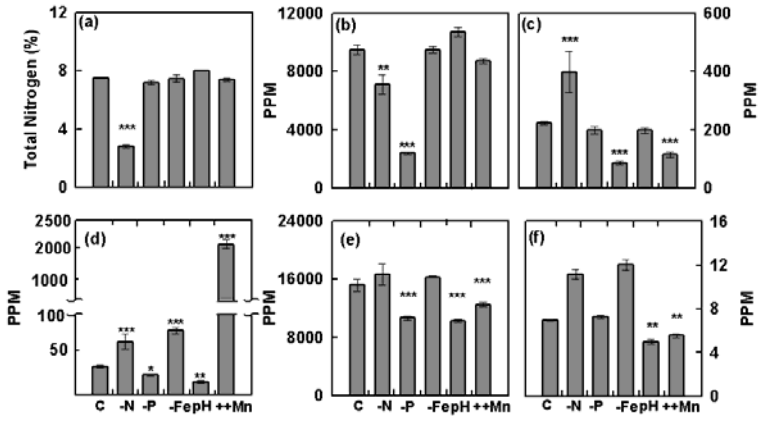

(a)
Total Nitrogen (%)
12
8
4
0
***
(b)
PPM
12000
8000
4000
0
**
***
(c)
600
400
200
0
PPM
***
***
***
(d)
PPM
2500
2000
1000
100
50
***
*
***
**
***
(e)
PPM
24000
16000
8000
0
***
***
***
(f)
16
12
8
4
0
PPM
**
**
C
-N
-P
-FepH
++Mn

Figure 2

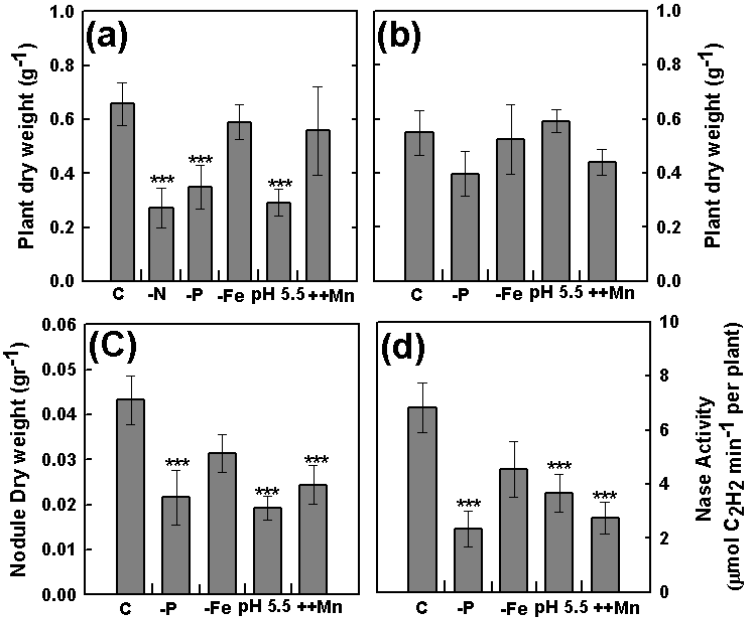

(a)
(b)
(C)
(d)
Plant dry weight (g-1)
Nodule Dry weight (gr-1)
Nase Activity (µmol C2H2 min-1 per plant)
C
-N
-P
-Fe
pH 5.5
++Mn
***

Figure 3

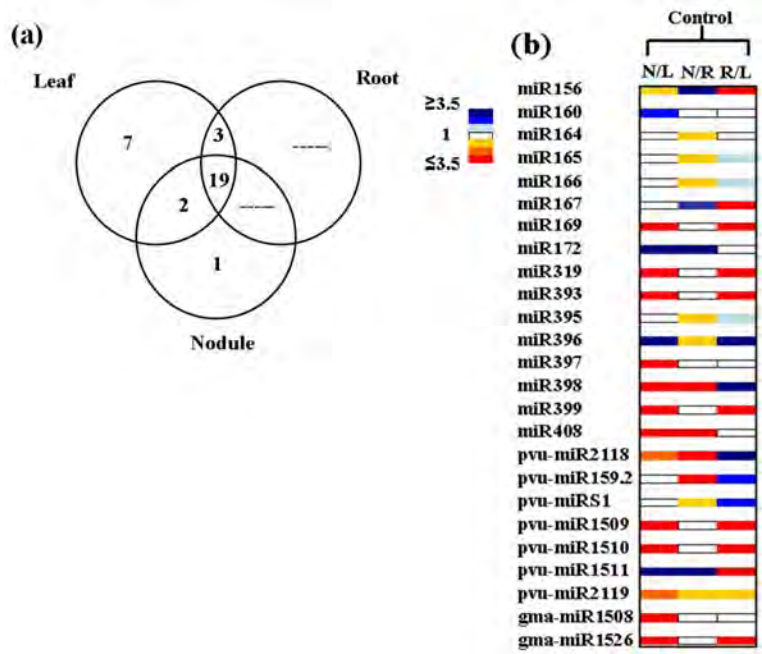

(a)
Leaf
Root
Nodule
7
3
19
2
1
(b)
Control
N/L N/R R/L
≥3.5
1
≤3.5
miR156
miR160
miR164
miR165
miR166
miR167
miR169
miR172
miR319
miR393
miR395
miR396
miR397
miR398
miR399
miR408
pvu-miR2118
pvu-miR159.2
pvu-miRS1
pvu-miR1509
pvu-miR1510
pvu-miR1511
pvu-miR2119
gma-miR1508
gma-miR1526

Figure 4

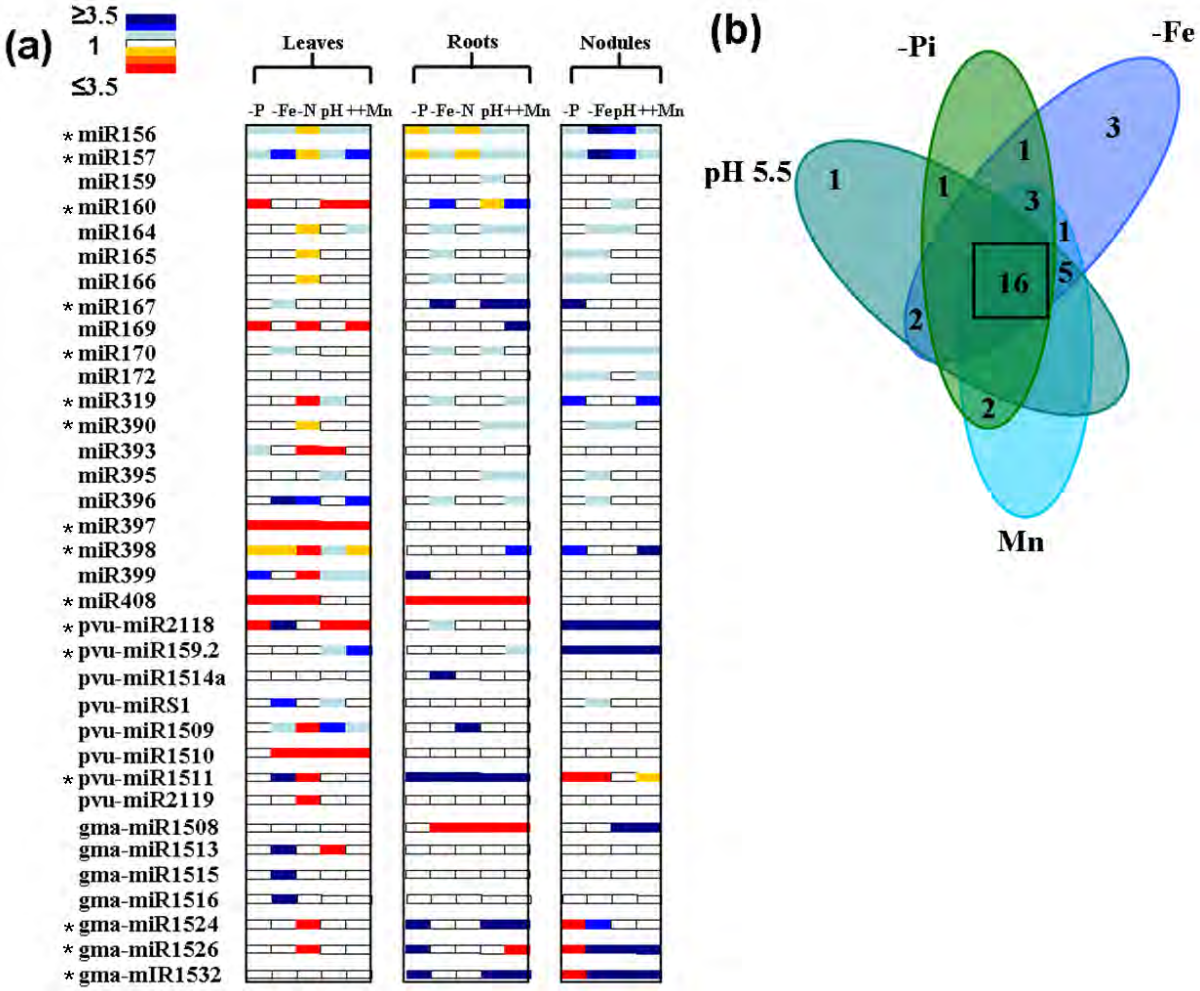

(a)
≥3.5
1
≤3.5
Leaves
Roots
Nodules
-P -Fe-N pH+ +Mn
-P -Fe-N pH+ +Mn
-P -FepH+ +Mn
* miR156
* miR157
miR159
* miR160
miR164
miR165
miR166
* miR167
miR169
* miR170
miR172
* miR319
* miR390
miR393
miR395
miR396
* miR397
* miR398
miR399
* miR408
* pvu-miR2118
* pvu-miR159.2
pvu-miR1514a
pvu-miRS1
pvu-miR1509
pvu-miR1510
* pvu-miR1511
pvu-miR2119
gma-miR1508
gma-miR1513
gma-miR1515
gma-miR1516
* gma-miR1524
* gma-miR1526
* gma-mIR1532
(b)
-Pi
-Fe
pH 5.5
Mn
1
1
1
3
3
1
16
5
2
2

Figure 5

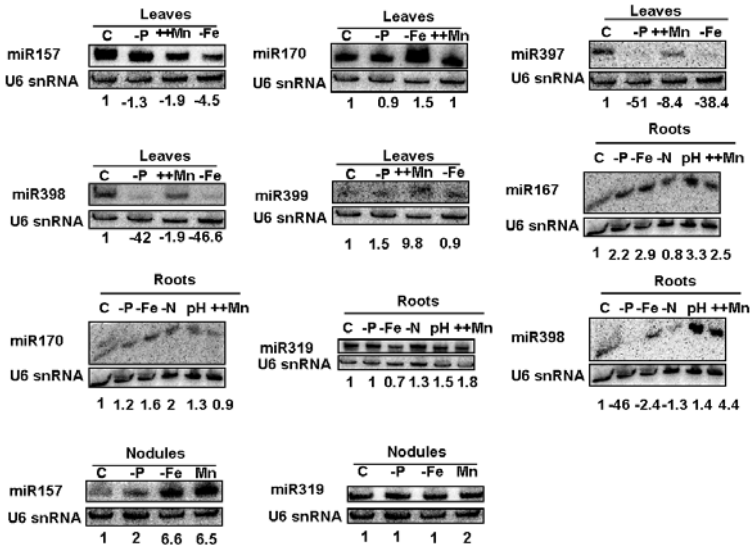
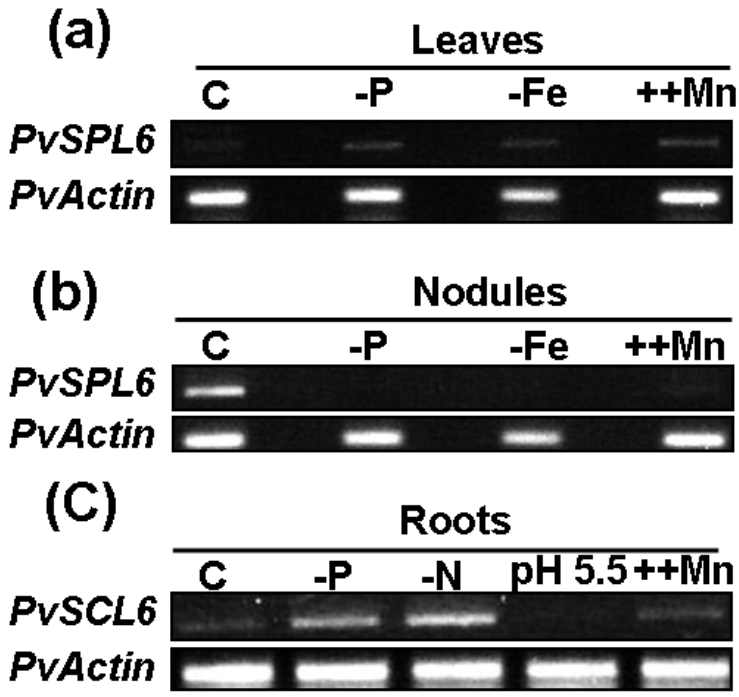

Leaves
C -P ++Mn -Fe
miR157
U6 snRNA
1 -1.3 -1.9 -4.5
Leaves
C -P -Fe ++Mn
miR170
U6 snRNA
1 0.9 1.5 1
Leaves
C -P ++Mn -Fe
miR397
U6 snRNA
1 -51 -8.4 -38.4
Leaves
C -P ++Mn -Fe
miR398
U6 snRNA
1 -42 -1.9 -46.6
Leaves
C -P ++Mn -Fe
miR399
U6 snRNA
1 1.5 9.8 0.9
Roots
C -P -Fe -N pH ++Mn
miR167
U6 snRNA
1 2.2 2.9 0.8 3.3 2.5
Roots
C -P -Fe -N pH ++Mn
miR170
U6 snRNA
1 1.2 1.6 2 1.3 0.9
Roots
C -P -Fe -N pH ++Mn
miR319
U6 snRNA
1 1 0.7 1.3 1.5 1.8
Roots
C -P -Fe -N pH ++Mn
miR398
U6 snRNA
1 -46 -2.4 -1.3 1.4 4.4
Nodules
C -P -Fe Mn
miR157
U6 snRNA
1 2 6.6 6.5
Nodules
C -P -Fe Mn
miR319
U6 snRNA
1 1 1 2

Figure 6


(a)
Leaves
C
-P
-Fe
++Mn
PvSPL6
PvActin
(b)
Nodules
C
-P
-Fe
++Mn
PvSPL6
PvActin
(C)
Roots
C
-P
-N
pH 5.5
++Mn
PvSCL6
PvActin

Supporting Information

Supporting Information Table S1: miRNA sequences used to synthesize oligonucleotides for miRNA-macroarrays printing and for miRNA-blot probes.

Supporting Information Table S2 Target mRNA of nutrient deficiency responsive- miRNAs: annotation, designed primers, and sRT-PCR conditions used for expression analysis. Sequences were obtained from Arenas-Huertero *et al.,* 2009.

Supporting Information Table S3 Statistical analysis of miRNAs-macroarray experiments.

Supporting Information Table S4 Organ-specific miRNAs from *P. vulgaris* plants grown under optimal nutritional conditions. Data of miRNAs in each category represent the mean from three biological replicates.

Supporting Information Table S5 miRNAs expressed in different nutritional stress conditions: miRNAs expressed in all tested treatments; miRNAs expressed in -P, -Fe, and ++Mn; miRNAs expressed in -Fe, pH 5.5, and ++Mn; miRNAs expressed in -P and -Fe; miRNAs expressed in -P and pH 5.5; miRNAs expressed in -Fe and pH 5.5. The data indicate the expression ratios (treatment/control) and are the mean of three biological replicates from different organs. TND indicates absent in the treatment but present in the control. CND indicates absent in the control but present in the treatment. NS indicates no statistical difference. ND indicates no detected in both conditions.

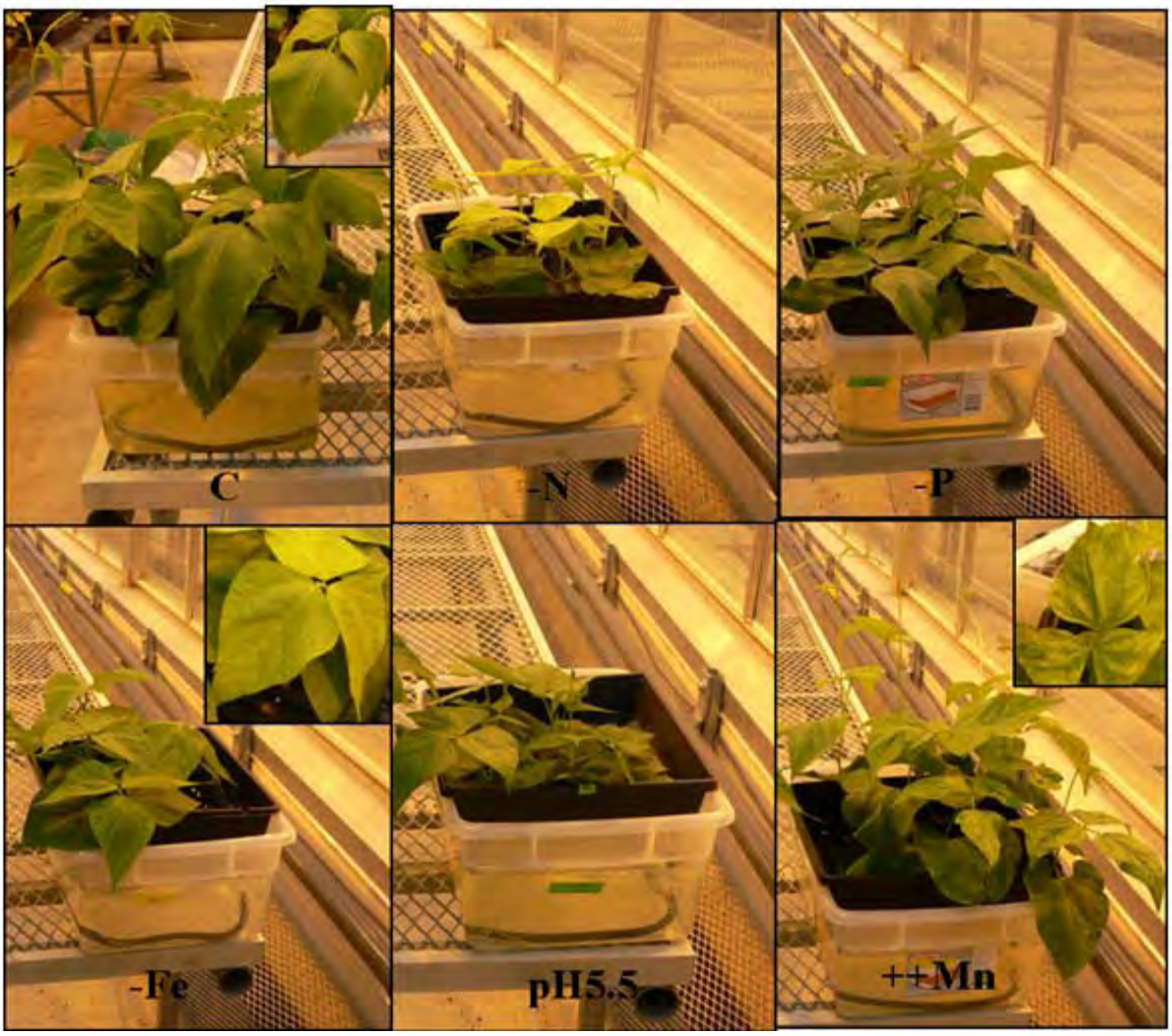
Supporting Information Figure 1 Visible symptoms of the different nutritional stresses.

Supporting Information Table S2. Target mRNA of nutrient deficiency responsive- miRNAs: annotation, designed primers, and sRT-PCR conditions used for expression analysis

EST/TC	GB #	Annotation	BLASTX E-Value	Designation	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Annealing Tm/Cycles
----------	FE707266.1	(B8Y9B2) Squamosa- promoter binding protein	6.00E-65	*SPL*	CAGACGCACACACCTGGGAATGG	AGGATAAAAATCAGAATTCATAGGA	290	58^0C/30*
TC3389	----------	(O81316) Scarecrow-like protein	2.00E-61	*SCL*	GACTTCATTCAAATGCCAACAACAG	TACAGCTTCACAATCACGAAGTGG	435	60^0C/25

TC No. Tentative consensus sequences assignment /DFCI Common_Bean_Gene_Index, version 2.0) The gene sequences were compared to the UniProt protein database (version August 2007) using BLASTX algorism. * The same conditions were used for a second round of PCR.

Supporting Information Figure S1. Visible symptoms of the different nutritional stresses.



VII. 7 Material y Métodos para el análisis de las respuestas de frijol a la toxicidad por Mn^{+2}.

Material vegetal y condiciones de crecimiento

Para este estudio se utilizó el cultivar mesoamericano de *Phaseolus vulgaris* "Negro Jamapa 81". Las plantas fueron crecidas en un invernadero con condiciones ambientales controladas (26-28 ^{0}C, 16 horas de fotoperíodo). Las semillas fueron esterilizadas superficialmente y germinadas en toallas de papel húmedas y estériles a 25 ^{0}C durante 2 días. Los germinados de 2 días de edad fueron sembrados en macetas con vermiculita húmeda y estéril e inoculados con *Rhizobium tropici* CIAT899. Después de 5 días de crecimiento los cotiledones fueron removidos y las macetas fueron hidratadas 3 veces a la semana con la solución nutritiva Summerfield (Summerfield *et al*., 1977) sin nitrógeno. Para condiciones control, la solución nutritiva no fue suplementada con exceso de Mn^{+2}, mientras que en los diferentes tratamientos la solución nutritiva fue suplementada con 0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM, 1 mM o 2 mM de $MnCl_2$. Después de evaluar algunos parámetros fisiológicos, se eligieron las condiciones de 0.2 mM (toxicidad moderada [M]) y 1 mM (toxicidad extrema[E]) de Mn^{+2} para el análisis del transcriptoma de nódulos de frijol sometidos a este estrés. Los nódulos fueron colectados e inmediatamente congelados en N_2 líquido y almacenados a -80 ^{0}C hasta su uso.

Determinación de la actividad de nitrogenasa y de los niveles de Mn^{+2} y Fe^{+2}.

La actividad de la nitrogenasa fue determinada por medio del ensayo de reducción de acetileno (ARA) en raíces nodulas de 15 días de tratamiento como reporta Mendoza *et al*., (1995). Los nódulos utilizados en este ensayo fueron colectados y secados a 50 ^{0}C durante 2 días para posteriormente obtener su peso seco y determinar los niveles de Mn^{+2} y Fe^{+2}. Para determinar los niveles de Mn^{+2} y Fe^{+2} 0.2 gr de nódulos secos fueron digeridos en HNO_3 concentrado durante 2 horas a 250 ^{0}C. Las muestras digeridas fueron diluídas en agua desionizada y se procedió a determinar por separado la concentración de Mn^{+2} y Fe^{+2} en un espectrofotómetro de absorción atómica PYE UNICAM SP 192.

Microscopía

Los nódulos de plantas sometidas a toxicidad moderada y extrema de Mn^{+2}, fueron colectados y fijados inmediatamente como reporta Trepp *et al*., (1999). Los nódulos embebidos fueron seccionados en cortes de 7 μm y colocados en postaobjetos cubiertos con poly-L-lys. Después de la eliminación de la parifina con xileno, los cortes fueron teñidos con una solución de azúl de metileno, deshidratados y montados con Permount (Fisher Scientific, Pittsburg). Los cortes fueron analizados en el microscopio y fotografiados con una cámara digital. Para los cortes en fresco, los nódulos fueron colectados en agua fría, posteriormente, sin ningún fijador, a cada nódulo se le hizo un corte transversal con una navaja y fueron analizados en un microscopio estereoscópico y fotografiados con una cámara digital.

Determinación de la actividad de la CAT, GPX y SOD.

Una muestra (0.5 gr) de los nódulos congelados a -80 ^{0}C fue macerada en un mortero frío y con 2 ml de buffer de homogenización (K_2PO_4 pH7.0 50mM; EDTA 50 mM, Ácido D-isoascórbico 1mM, PVP 0.2 % y Triton X-100). El macerado fue centrifugado a 10,000 g durante 10 minutos a 4 ^{0}C. El sobrenadante se utilizó para determinar la actividad de catalasa (CAT) y de la guayacol peroxidasa (GPX). Para la actividad de la superoxido dismutasa (SOD), los nódulos fueron macerados en un mortero frío y con buffer de K_2PO_4 2 mM pH7.8, Na_2EDTA 0.1 mM y 1% de PVP insoluble. El macerado fue centrifugado a 10,000 g durante 10 minutos a 4 ^{0}C.

La actividad de CAT fue determinada en buffer de fosfatos 50 mM pH 7.0 mediante la producción de óxigeno a partir del H_2O_2 (33 mM), contenido en el buffer de reacción. La producción de oxígeno fue monitoreada con un Oxímetro Gylson IC-OXY con electrodo tipo Clark. La actividad específica de CAT es reportada como: μmol O_2 Evolved/min^{-1}/ mg^{-1} of protein

La actividad de GPX fue determinada espectrofotométricamente a 25 ^{0}C. El buffer de reacción contenía: K_2PO_4 50 mM pH7.0, 2mM/L H_2O_2 y guayacol 2.7 mM. La reacción fue inciada con la adición del extracto enzimático que contenía 5 μg de proteína. La formación de tetraguayacol fue medida a 470 nm (ε= 26.6 $mmol/L^{-1}cm^{-1}$). La actividad de esta enzima fue reportada en Unidades Enzimáticas/mg de proteína (EU/mg of protein)

Para determinar la actividad de SOD se uso 1 ml de la mezcla de reacción que contenía: K_2PO_4 50 mM pH 7.8, L-methionina 30 mg), azul de tetrazolio 1.4mg, Triton X-100 y 20 μl de extracto enzimático (40 μg de proteína) o de buffer (control). Esta mezcla fue iluminada durante 10 minutos con 2 lámparas fluorescentes de 20W. El cambio de la absorbancia, debido a la formación o a la inhibición de la formación de formazan, fue determinado a 560 nm. El incremento de la absorbancia en la ausencia de cualquier extracto enzimático fue considerado como 100%. La actividad de esta enzima fue reportada en EU/mg of protein. En este estudio, una unidad enzimática de SOD es equivalente al 50% de la inhibición de la formación de formazan.

Aislamiento de RNA total e hibridación de macroarreglos

Se aisló RNA total a partir de 0.5 g de nódulos congelados de plantas control o sometidas a toxicidad moderada o extrema de Mn^{+2} (Valdés-López *et al*., 2008). Se sintetizaron sondas de cDNA radiactivo a partir 30 μg de RNA total de nódulos control o tratados con toxicidad de Mn^{+2}. Estas sondas se usaron para hibridar los macroarreglos impresos con 7, 200 EST provenientes de nódulos control de frijol común. Las condiciones de hibridación y lavado de los macroarreglos están reportados en Ramírez *et al*., (2005). Se hibridaron 3 macroarreglos con cDNA radiactivo proveniente de tres réplicas biológicas de nódulos control o tratados con toxicidad de Mn^{+2} (toxicidad moderada o extrema).

Los macroarreglos hibridados fueron expuestos durante 2 días al sistema Phosphor Screen (Amersham). Las pantallas del sistema Phosphor Screen (Amersham) fueron escaneadas en un Storm 860 Gel and Blot Imaging System (GE Healthcare, Buckinghamshire, UK). La intensidad de la fluorescencia de cada punto fue cuantificada usando el software Array-Pro Analyzer (Media Cybernetics). Se realizó el análisis de regresión lineal en cada replica de cada tratamiento. Solo aquellas replicas para las cuales la regresión lineal pudo explicar al menos el 80% de la variación ($r^2 \geq 0.8$) fueron consideradas. Los datos fueron ajustados con el promedio de la intensidad del

"housekeeping gene" que codifica para la enzima ubiquitina-conjugasa (TC8137). Los datos normalizados fueron utilizados para obtener las proporciones de expresión: M/C y E/C. Para cada uno de las ESTs del macroarreglo se realizó la prueba *t* de Student con un *P*-value ≤ 0.05. Unicamente los genes con un nivel de expresión ≥ 2 y con un *P*≤0.05 fueron considerados. Para identificar los genes que respondieron a los dos niveles de toxicidad por Mn^{+2}, se aplicó el análisis de flor a los datos obtenidos del análisis de macroarreglos.

RT-PCR semicuantitativo

El enfoque de RT-PCR semicuantitativo (sRT-PCR) fue utilizado para verificar los datos de expresión obtenidos del análisis de macroarreglos y para determinar los niveles de expresión de los blancos de los miRNAs miR157 y miR319. El aislamiento de RNA total y la síntesis de cDNA fueron realizados como se reportó en Valdés-López *et al*., (2008). Los productos obtenidos de sRT-PCR se resolvieron en géles de agarosa al 1.5% y se visualizaron con bromuro de etidio. La intensidad de cada producto de sRT-PCR fue determinada con el software ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA, USA). Para cada gene y nivel de toxicidad de Mn^{+2} se hicieron tres réplicas con cDNA proveniente de tres réplicas biológicas. Las secuencias y condiciones de PCR están descritas en el Anexo VII. 8.

Northern-Blot para miRNAs

Para el análisis de la expresión de miRNAs mediante Northern blot, se utilizaron muestras de RNA total de nódulos control y de los diferentes niveles de toxicidad de Mn^{+2} y se corrieron en geles de poliacrilamida 15%/UREA 8M/TBE 1X. Los geles fueron electro-transferidos a membranas Hybond N+ (GE Healthcare) y fijados 2 veces con luz UV. Las hibridaciones fueron realizadas a 42 ^{0}C durante 15 hrs en el buffer UltraHyb-solution (Ambion, Austin, TX, USA). Las membranas hibridadas fueron lavadas dos veces en 2X SSC/0.1%SDS durante 30 minutos cada vez, y después fueron expuestas al sistema Phosphor Screen (GE Healthcare, UK) para determinar su intensidad como se describió previamente. Se utilizaron los oligonucleótidos sintéticos de DNA con la secuencia antisentido correspondiente a miR157 (GTGCTCTCTATCTTCTGTCAA) y miR319 (GGGAGCTCCCTTCAGTCCAA), como sondas marcadas radiactivamente con [γ-^{32}P]-ATP (Perkin-Elmer, Boston, MA) y T4 PNK (New England Biolabs) en el extremo 5'. Previo a la adición de las sondas al buffer de hibridación, éstas fueron purificadas con las columnas Quick spin oligo columns (Roche). El oligonucletido sintético de DNA complementario a U6 snRNA se uso como sonda para control de carga.

Anexo VII.8: Selected Mn^{+2} toxicity-responsive common bean genes: annotation, designed primers and sRT-PCR conditions used for expression analysis.

EST/TC #GB	Annotation	Blast-X E-value	Designation	Primer Forward (5'-3')	Primer Reverse (5'-3')	Product size (bp)	Annealing Tm/Cycles
TC13275	(P08297) Early nodulin 75	1.00E-130	*PvENOD75*	GAATACCAA CCTCCTCATGAGAAG	GTGATTTCTCATAAGGAGGTTGGT	400 bp	60^0C/23
TC1395	(P04770) Glutamine synthetase	0	*PvGs*	TGAATCTCAGGATTCAGAGAGAAA	ATAGACACAGGCTTTGTAATGTGC	587bp	58^0C/23
TC10827	(Q02917) Early nodulin-55-2	7.00E-71	*PvENOD55*	TCAGATATTGAATGAACATGGGTGT	TTGAACTACCAGTAACACCGTGGTT	559bp	58^0C/18
TC10979	(O24088) MtN24 protein	4.00E-42	*PvMt24*	CAAAAGAACTCTTGATCACACATTCA	AATGAATTTGAAAGTCTTGTAGAGATG	575bp	58^0C/23
TC12109	(O23190) MAP3K-like protein	2.00E-83	*PvMAPK*	CGAAATGGTGTGAATAAAGTGTTTAA	AAGTATGATATCTTCATCATATCCAA	648bp	58^0C/23
TC11312	(Q41712) Ascorbate Peroxcidase	1.00E-139	*PvAPX*	TAAGCCATGGGAAAGTCTTACCCCTC	CCTTGAAGTATGAGTTGTCAAAAATA	671bp	58^0C/23
TC10138	(Q2B5BL1) SNARE	1.00E-114	*PvSNR*	GTCTTTCTCTTTCTCTCCTCAAGATA	AAGCATGTAATTCAACAAACTCTTTC	569bp	60^0C/30
TC9755	(B9RFD9) RNA binding protein	4.00E-66	*PvRbP*	GTTTTTGATGGCGGCGTCTTCTTCTT	GTTCAGCAGTTCATTTCTCTTGCTTT	553bp	58^0C/23
TC9243	(Q9FFB3) Vacuolar protein sorting-associated protein	1.00E-104	*PvVPS*	GTCGCAACATTGAGCGCCAAATTC	ATTAACCTGTCAACATCTCAATCTC	679bp	58^0C/23
TC9777	(Q8GVP6) Pyrrolidone carboxyl peptidase-like protein	3.00E-84	*PvPC*	GAGTAGATAAGACTCGTGGATGTC TC	AATGATAGTAAACATAGTTGCACACA	643bp	58^0C/23
FE707266.1	(B8Y9B2) Squamosa- promoter binding protein	6.00E-65	*PvSPL*	CAGACGCACACACCTGGGAATGG	AGGATAAAAATCAGAATTCATAGGA	290bp	58^0C/30*
TC3389	(O81316) Scarecrow-like protein	2.00E-61	*PvSCL*	GACTTCATTCAAATGCCAACAACAG	TACAGCTTCACAATCACGAAGTGG	435bp	60^0C/30*

TC No. Tentative consensus sequences assignment /DFCI Common_Bean_Gene_Index, version 3.0) The Genes Sequences were compared to the UniProt protein database (version August 2007) using BLASTX algorism. * The same conditions were used for a second round of PCR.