UNIVERSIDAD DE CÓRDOBA

FACULTAD DE CIENCIAS

DEPARTAMENTO DE BOTÁNICA, ECOLOGÍA Y FISIOLOGÍA VEGETAL

Accumulation of ureides in response to water deficit in *Phaseolus vulgaris* and characterization of PRAT and XDH, two key enzymes in ureide synthesis

Tesis Doctoral

Inmaculada Coleto Reyes

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TITULO: *Acumulación de ureidos en respuesta al déficit hídrico en Phaseolus vulgaris y caracterización de la PRAT y la XDH, dos enzimas clave en la síntesis de ureidos. /Acumulation of ureides en response to water defecit in Phaseolus vulgaris and characterization of PRAT and XDH, two key in ureides synthesis.*

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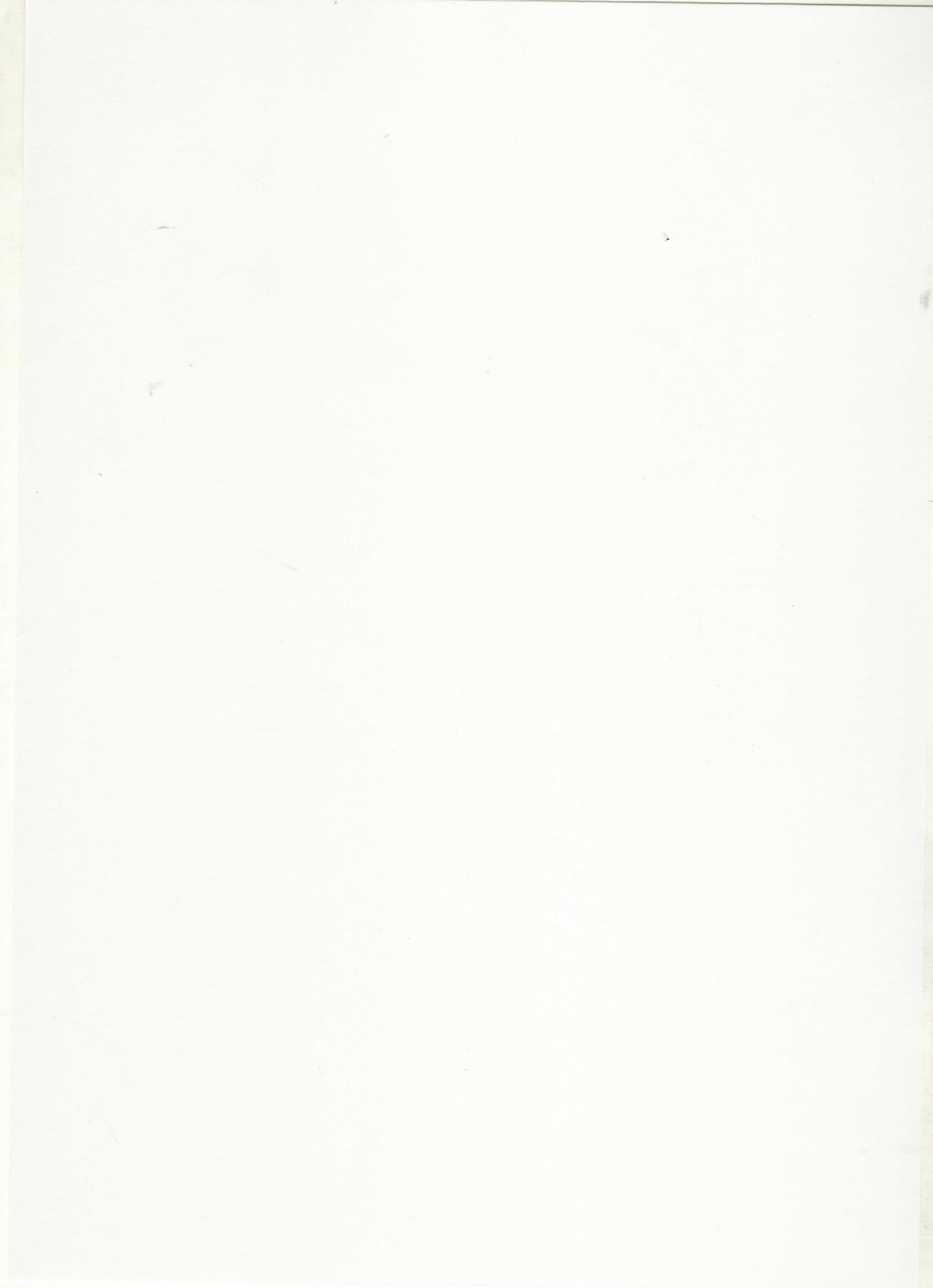
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Accumulation of ureides in response to water deficit in *Phaseolus vulgaris* and characterization of PRAT and XDH, two key enzymes in ureide synthesis

Memoria presentada por Inmaculada Coleto Reyes para optar

al grado de Doctor por la Universidad de Córdoba

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TÍTULO DE LA TESIS: ACCUMULATION OF UREIDES IN RESPONSE TO WATER DEFICIT IN *PHASEOLUS VULGARIS* AND CHARACTERIZATION OF PRAT AND XDH, TWO KEY ENZYMES IN UREIDE SYNTHESIS

DOCTORANDO/A: INMACULADA COLETO REYES

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

El trabajo de Tesis Doctoral de Dña. Inmaculada Coleto Reyes se ha llevado a cabo en el Departamento de Botánica, Ecología y Fisiología Vegetal, en el grupo "Biotecnología de plantas superiores y algas verdes" (BIO-115). Durante la realización de la Tesis, la doctoranda ha utilizado con éxito numerosas técnicas propias de la Fisiología y Bioquímica Vegetal, así como de Biología Molecular y Genética, necesarias para el desarrollo experimental de la misma. La doctoranda ha demostrado una gran capacidad de trabajo, así como una gran vocación y excelente aptitud ante los retos de la experimentación científica, y ha adquirido una sólida formación, no solo en el área de estudio abordada en la Tesis, sino de carácter más general, que la capacitan para abordar proyectos de investigación con total independencia, abarcando desde el diseño de los experimentos hasta el análisis y puesta en valor de los resultados obtenidos, así como la organización de los mismos para su difusión nacional e internacional. El trabajo realizado ha permitido indagar en el origen de los ureidos que se acumulan en *Phaseolus vulgaris,* en condiciones de estrés hídrico, mostrando que dichos ureidos proceden de la degradación de los nucleótidos de purina previamente presentes en la célula y no de purinas sintetizadas *de novo*. Durante el proceso, se han caracterizado a nivel molecular y funcional dos enzimas cruciales para la síntesis de los ureidos, como son la glutamina fosforibosilpirofosfatoamidotransferasa y la xantina deshidrogenasa.

Los resultados obtenidos se han comunicado en varios congresos nacionales e internacionales, y han dado lugar una publicación, así como a otras dos, todavía en proceso de edición.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 10 de noviembre de 2015

Firma del/de los director/es

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Parte de los resultados que se presentan en esta Tesis Doctoral han dado lugar a la siguiente publicación:

- **Coleto**, I., Pineda, M., Rodiño, A.P., de Ron, A.M., Alamillo, J.M. (2014) Comparison of inhibition of N_2 fixation and ureide accumulation under water deficit in four common bean genotypes of contrasting drought tolerance. *Annals of Botany* 113: 1071-1082.

Asimismo, se han presentado en los siguientes congresos:

- Alamillo, J.M., Díaz-Leal, J.L., **Coleto**, I., Quiles, F.A., Pineda, M. Regulación del metabolismo de ureidos por ABA y otras señales de estrés abiótico. XXXIII Congreso de Bioquímica y Biología Molecular (SEBBM). Córdoba (España). 2010.
- **Coleto**, I., Pineda, M., y Alamillo, J.M. Efecto del déficit hídrico sobre la fijación de nitrógeno y la síntesis de ureidos en cultivares sensibles y tolerantes de *Phaseolus vulgaris.* XIX Reunión de la Sociedad Española de Fisiología Vegetal (SEFV). Castellón de la Plana (España) 2011.
- **Coleto**, I., Pineda, M., Alamillo, J.M. Análisis del metabolismo de ureidos durante el estrés hídrico en *Phaseolus vulgaris.* I Congreso Científico de investigadores en formación en Agroalimentación. Córdoba (España). 2012.
- **Coleto**, I., Pineda, M., Alamillo, J.M. Relación de la sensibilidad de la fijación de nitrógeno a la sequía y la acumulación de ureidos en varios cultivares de *Phaseolus vulgaris.* IV Jornadas de la Asociación Española de Leguminosas (AEL) – V Seminario de Judía. Pontevedra (España). 2012.
- **Coleto**, I., Pineda, M., Alamillo, J.M. Caracterización molecular y bioquímica de los mecanismos de acumulación de ureidos en respuesta a la sequía en *Phaseolus vulgaris.* XI Reunión nacional del metabolismo del

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- Alamillo, J.M., **Coleto**, I., Díaz-Lea,l J.L., Gálvez Valdivieso, G., Pineda, M. Nuevas perspectivas sobre la función fisiológica de la acumulación de ureidos en condiciones de estrés. V Reunión Nacional de la Red Española de Estrés Abiótico de las Plantas (REAP). Murcia (España). 2012.

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- **Coleto**, I., Trenas, A.T., Pineda, M., Alamillo, J.M. Molecular and physiological characterization of *Phaseolus vulgaris* phosphoribosylpyrophosphate amidotransferase (PRAT). XIII Congreso Luso-Español de Fisiología Vegetal. Lisboa (Portugal). 2013.

- **Coleto**, I., Trenas, A.T., Erban, A., Kopka, J., Pineda, M., Alamillo, J.M. Molecular and functional characterization of PRAT gene family from *Phaseolus vulgaris.* XII Reunión Nacional del Metabolismo del Nitrógeno. Bilbao (España). 2014.

- Coleto, I., Pineda, M., Alamillo, J.M. Caracterización funcional de la glutamina fosforibosilpirofosfato amidotransferasa (PRAT) y la xantina deshidrogenasa (XDH) de *Phaseolus vulgaris*: dos enzimas clave en la síntesis de ureidos. II Congreso Científico de investigadores en formación en Agroalimentación. Córdoba (España). 2014.

- **Coleto**, I., Pineda, M., Alamillo, J.M. Regulation of *Phaseolus vulgaris* (common bean) ureide metabolism during drought stress. XIV Congreso Luso-Español de Fisiología Vegetal. Toledo (España). 2015

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ABBREVIATIONS

AAH: Allantoate amidohydrolase

ABA: Abscisic acid

ACC: 1-aminocyclopropane-1-carboxylic acid

ALN: Allantoinase

AMP: Adenosine monophosphate

ANA: Aparent nitrogenase activity

Asn: Asparagine

BCIP: 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt

BNF: Biological nitrogen fixation

BSTFA: N,O-bis (trimethylsilyl)trifluoroacetamide

Cys: Cysteine

DW: Dry weight

FAD: Flavin adenin dinucleotide

DOC: Deoxycholic acid

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

EST: Expressed sequences tag

FeCy: Ferricyanide

FW: Fresh weight

GA: Gibberellic acid

GAR: Formylglycinamide ribonucleotide

GC-MS: Gas chromatography-Mass spectrometry

GDH: Glutamate deshydrogenase

Gln: Glutamine

GMD: Golm metabolome database

GMP: Guanosine monophosphate

GSNO: S-nitrosoglutathione

HA: Hemagglutinin

HIU: 5-hidroxiisourate

IG: Immunoglobuline

IMP: Inosine monophosphate

JA: Jasmonic acid

Moco: Molybdenum cofactor

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD: Nicotinamide adenine dinucleotide

NADHox: NADH oxidase

NBT: Nitroblue tetrazolium

NO: Nitric oxide

OHCU: 2-oxo-4-hidroxi-4-carboxi-5-ureidoimidazolina

PAGE: Polyacrilamide gel electrophoresis

PMS: Phenazine methosulfate

PPD: Phenylphosphorodiamidate

PRA: Phosphoribosylamine

PRAT: Phosphoribosylpyrophosphate amidotransferase

PRPP: Phosphoribosylpyrophosphate

RACE: Rapid amplification of cDNA ends

RNAi: Interferent ribonucleic acid

RNS: Reactive nitrogen species

ROS: Reactive oxigen species

RWC: Relative water content

SA: Salicylic acid

SNAP: S-nitroso-N-acetylpenicillamine

SNP: Sodium nitroprusside

SOD: Superoxide dismutase

SWC: Soil water capacity

Tea: Triethanolamine hydrochloride

TNA: Total nitrogenase activity

TW: Turgid weight

UO: Urate oxidase

XDH: Xanthine dehydrogenase

XMP: Xantosine monophosphate

XO: Xanthine oxidase

XOR: Xanthine oxidoreductase

RESUMEN

Los ureidos alantoína y alantoato son las formas mayoritarias en las que se almacena y transporta el nitrógeno fijado en los nódulos de las leguminosas tropicales o de tipo ureídico como la soja (*Glycine max*) y la judía (*Phaseolus vulgaris*). En las leguminosas ureídicas, el déficit hídrico produce la inhibición de la fijación de nitrógeno, a la vez que aumenta el contenido de ureidos en las hojas. La síntesis de ureidos se lleva a cabo por el catabolismo oxidativo de purinas que pueden haber sido sintetizadas *de novo* o proceder del reciclaje de los nucleótidos de purina. En este trabajo, se ha llevado a cabo un análisis del metabolismo de los ureidos en variedades de *P. vulgaris* con distinto grado de tolerancia a la sequía. La determinación del contenido de ureidos en los distintos genotipos de judía sometidos a estrés hídrico mostró que la acumulación de ureidos se correlacionaba con el grado de tolerancia a la sequía de los mismos, siendo mayor en los más sensibles. Además, la acumulación de ureidos se debía a la activación de su síntesis en tejidos diferentes a los nódulos. Para analizar la procedencia de los ureidos acumulados durante el estrés hídrico, se han estudiado las enzimas xantina deshidrogenasa (XDH) y glutamina fosforribosilpirofosfato amidotransferasa (PRAT), cruciales en la regulación de la oxidación y en la síntesis *de novo* de purinas, respectivamente. Se ha aislado la secuencia codificante y la región promotora del gen *XDH* de *P. vulgaris*, en la que se han encontrado diversos motivos de regulación por estrés. Los análisis de expresión y de actividad mostraron un aumento del nivel de PvXDH en respuesta a la sequía y a tratamientos con diferentes fitohormonas relacionadas con estreses abióticos o bióticos. Además de la actividad deshidrogenasa, la enzima PvXDH es capaz de catalizar la oxidación de la xantina y el NADH por oxígeno molecular. Sin embargo, solamente la actividad deshidrogenasa aumentó por el estrés. Curiosamente, el óxido nítrico (ON) produjo una inhibición de la actividad XDH en las hojas pero no en los nódulos de judía y la inhibición en hojas se impidió por ácido úrico añadido. Cuando se bloqueó la expresión del gen *PvXDH* por

RNAi en nódulos, el ON también inhibió la actividad XDH; lo que podría deberse a una disminución de ácido úrico, producto de la XDH. Asimismo, se han aislado y analizado las secuencias de los tres genes que codifican PRATs en *P. vulgaris* y se ha abordado la caracterización bioquímica de las proteínas PRAT, que se purificaron tras su sobreexpresión transitoria en hojas de *Nicotiana benthamiana*. El análisis de los patrones de expresión de dichos genes mostró que *PvPRAT1* y *PvPRAT2* se encargan de mantener el nivel de los nucleótidos de purina preferentemente en las hojas y tejidos aéreos de la planta. En cambio, *PvPRAT3* tiene una función predominante en los nódulos y su expresión y actividad se correlacionaron con la fijación de nitrógeno. El análisis metabolómico de raíces transgénicas en las que se bloqueó la expresión del gen *PvPRAT3* mediante RNAi mostró una disminución en la concentración de ureidos y un aumento en la concentración de aminoácidos en las raíces silenciadas, lo que sugirió que PvPRAT3 podría tener un papel determinante en la diferenciación entre las leguminosas ureídicas y amídicas. En las secuencias promotoras de los genes *PvPRAT* se encontraron varios motivos de regulación por estrés; sin embargo, el estrés hídrico no indujo cambios en la expresión ni en la actividad de las proteínas PvPRAT. En conjunto, los resultados obtenidos demuestran que en condiciones de estrés se produce una activación de la oxidación de purinas, en las que está implicada la XDH, pero no una activación de su síntesis *de novo*.

SUMMARY

The ureides allantoin and allantoate are the mayor forms in which the nitrogen fixed in the nodules is stored and exported in tropical or ureidic legumes such as the soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*). In ureidic legumes, nitrogen fixation is inhibited under water deficit, but the ureide content of leaves increases under these conditions. Ureide synthesis takes place by the oxidative degradation of purine bases, whose origin may be the *de novo* synthesis or the remobilization of preexisting purine nucleotides. In this work, an analysis of the ureide metabolism has been performed in several varieties of *P. vulgaris* with different degree of drought tolerance. The determination of the ureide concentration in different common bean genotypes subjected to water stress showed that the accumulation of ureides correlates with the degree of drought sensitivity of the varieties, being higher in the sensitive varieties than in the tolerant ones. Moreover, ureides accumulation was due to the activation of their synthesis in tissues other than nodules. To determine the origin of ureides accumulated during water deficit, the enzymes xanthine dehydrogenase (XDH) and glutamine phosphoribosylpyrophosphate amidotransferase (PRAT), which are pivotal in the regulation of the oxidation and *de novo* synthesis of purines, respectively, have been studied. The coding sequence and the promoter region of the *XDH* gene from *P. vulgaris* have been isolated. Analysis of the sequences revealed the presence of several stress-related regulatory motifs in the proximal promoter sequence and expression and activity analyses showed an enhancement in the PvXDH level in response to drought and in response to treatments with phytohormones related to abiotic and biotic stresses. Besides the dehydrogenase activity, the PvXDH protein is also able to catalyze the oxidation of xanthine and NADH by molecular oxygen; however, only the dehydrogenase activity increased in response to stress. Interestingly, nitric oxide (NO) inhibited the XDH activity in leaves, but not in nodules of common bean, and the inhibition in leaves was blocked by exogenous uric acid. When the expression of *PvXDH* gene was

silenced by RNAi in nodules, NO inhibited XDH activity, which may be due to a decrease in the content of uric acid, product of XDH. Likewise, the coding sequences of PRATs in *P. vulgaris* have been isolated and analyzed, and PvPRAT proteins, which were purified after their transitory overexpression in leaves of *Nicotiana benthamiana*, have been characterized. The expression patterns of *PvPRAT* genes showed that *PvPRAT1* and *PvPRAT2* are responsible for maintaining the level of purine nucleotides preferably in leaves and aerial plant tissues. Instead, PvPRAT3 has a predominant role in nodules and its expression and activity were correlated with nitrogen fixation. Moreover, the metabolomic analysis of transgenic hairy roots, in which the expression of *PvPRAT3* was inhibited by using RNAi, showed a decrease in the ureide concentration and an increase in the amino acid concentration of silenced roots, suggesting that PvPRAT3 may have a determinant role in the differentiation of the ureidic legumes. On the other hand, although several stress-related regulatory motifs were found in the promoter sequences of *PvPRAT* genes, water stress did not induce changes nor in the expression neither in the activity of PvPRAT proteins. Taken together, these results show that the oxidation of purines, in which XDH is implicated, but not their synthesis *de novo*, is activated during stress conditions.

CAPÍTULO 1: INTRODUCCIÓN GENERAL

1. Importancia económica, agronómica y social de la judía común

Las leguminosas, grupo al que pertenece la judía común (*Phaseolus vulgaris* L.), son la familia de plantas cultivables más importante después de los cereales. Estas plantas suponen un 27% de la producción vegetal global (Smýkal y Konecná, 2014) y aportan el 33% de la proteína para consumo humano a nivel mundial (Graham y Vance, 2003). El cultivo de las especies del género *Phaseolus* se emplea para obtener semillas secas o frutos verdes para consumo humano y para la obtención de pienso y forraje para el ganado (Freytag y Debouck, 2002). Dentro del género *Phaseolus*, la judía común es la especie más importante, ocupando el 80% de la superficie total cultivada con especies de este género (Singh, 1992; Smil, 1999). Actualmente, es la segunda leguminosa más cultivada del mundo, después de la soja, y la principal leguminosa de grano para consumo humano (Broughton *et al*., 2003; Beebe, 2012). Es un alimento básico, fuente de proteínas (20-25% del peso total de la semilla), micronutrientes (principalmente hierro) y calorías para alrededor de 500 millones de personas en el mundo, principalmente de los países en desarrollo, donde es un componente importante de las dietas tradicionales (Singh, 2005). La cosecha mundial de judía es de aproximadamente 23 millones de toneladas al año. Asia y América son las principales regiones productoras, contribuyendo con un 44,6% y un 33,1% de la producción total, respectivamente (FAOSTAT, 2013). En Europa, la judía es un cultivo más minoritario y su producción se concentra alrededor del Mediterráneo (Beebe *et al*., 2013). El principal factor ambiental limitante del cultivo de judía es la sequía (Zahran, 1999). Aproximadamente, el 60% de la producción tiene lugar en zonas expuestas a periodos de sequía y que no disponen de sistemas de irrigación externa, lo que da lugar a pérdidas que pueden alcanzar el 80% de la producción (Broughton *et al*., 2003; Miklas *et al*., 2006).

CHAPTER 1: GENERAL INTRODUCTION

1. Economic, agronomic, and societal importance of common bean

The common bean (*Phaseolus vulgaris* L.) is a plant belonging to the family Fabaceae or Leguminosae. Legumes represent the second most economically important family of crop plants after cereals, accounting for 27% of world crop production and providing 33% of the dietary protein consumed by humans (Smýkal and Konecná, 2014). Farming of the species of the genus *Phaseolus* is used to obtain dry seeds or fresh pods for human consumption and also can be exploited as fodder for livestock (Freytag and Debouck, 2002). Among them, common bean is the most economically important specie, occupying the 80% of the earth surface cultivated with species of this genus (Singh, 1992; Smil, 1999) and, currently, it is the second most cultivated legume worldwide after soybean and the most important grain legume for direct human consumption (Broughton *et al*., 2003; Beebe, 2012). Common bean is a staple food and a source of dietary proteins (20-25% of total seed weight), micronutrients (mainly iron), calories and oil for over 500 million people worldwide, living mostly in developing countries, where it is an important component of traditional diets (Singh, 2005). The global bean harvest is approximately 23 million tons. Asia and America are the highest producer regions, representing 44.6% and 31.1%, respectively (FAOSTAT, 2013). In Europe, beans are a minoritary crop, being concentrated around the Mediterranean (Beebe *et al*., 2013). The major environmental factor that limits the productivity of common bean crop is drought (Zahran, 1999). Approximately a 60% production occurs in agricultural lands exposed to drought periods and that lack external irrigation systems, resulting in production losses that may reach up to 80% of reduction of potential yield (Broughton *et al*., 2003; Miklas *et al*., 2006).

2. *Phaseolus* como organismo experimental

El género *Phaseolus* forma parte de la tribu de leguminosas tropicales Phaseoleae, a la que también pertenecen especies como el caupí (*Vigna unguiculata*) y la soja (*Glycine max*). La tribu Phaseoleae está incluida en el clado Milletioides, que divergió hace aproximadamente 54,3 millones de años del clado Hologalenina, en el cual se incluyen la mayoría de las leguminosas de climas templados como el guisante (*Pisum sativum*) y la alfalfa (*Medicago sativa*) (Lavin *et al*., 2005).

Las variedades de judía común cultivadas en la actualidad son el resultado de un proceso de domesticación y de evolución a partir de dos acervos genéticos principales, que se originaron en condiciones naturales en Mesoamérica y en Los Andes a partir de un antecesor común que aún persiste (Gentry, 1969; Kaplan, 1981; Brücher, 1988). Las variedades resultantes se han agrupado en seis razas (Singh *et al*., 1991), tres originadas en América central (las razas Durango, Jalisco y Mesoamérica) y tres originadas en la zona de Los Andes (las razas Chile, Nueva Granada y Perú).

P. *vulgaris* es un organismo diploide, con 11 cromosomas (2n =22) y un genoma relativamente pequeño (650 Mpb). A diferencia de otras leguminosas de la tribu Phaseoleae, como la soja, el nivel de duplicaciones cromosómicas y de regiones repetitivas en su genoma es relativamente bajo, de manera que la mayoría de los locus son de copia única y las familias génicas tienden a ser pequeñas (Vallejos *et al*., 1992; Freyre *et al*., 1998; McClean *et al*., 2002).

3. Fijación biológica del nitrógeno

Generalmente, las plantas adquieren el nitrógeno a partir de las reservas disponibles en el suelo. Sin embargo, cuando estos nutrientes son escasos, algunas plantas son capaces de fijar el N_2 atmosférico a través de la simbiosis con bacterias del suelo, a las que en conjunto se denomina rizobios. La fijación biológica del nitrógeno (FBN) se encuentra en varias familias de plantas, pero es más frecuente en las leguminosas (Sprent, 2002).

2. *Phaseolus* as an experimental organism

The genus *Phaseolus* is a member of the tropical tribe Phaseoleae, to which also belong cowpea (*Vigna unguiculata*), and soybean (*Glycine max*). The Phaseoleae tribe is part of the Milletioid clade, which diverged 54.3 million years ago from the Hologalegina clade, which contains most temperate crop legumes, such as pea (*Pisum sativum*) and alfalfa (*Medicago sativa*) (Lavin *et al*., 2005).

The varieties of common bean currently cultivated are the result of the domestication and evolution from two major gene pools originated under natural conditions both in Mesoamerica and the Andes from a common ancestor still extant (Grentry, 1969; Kaplan, 1981; Brücher 1988). The varieties resultant are grouped into six races (Singh *et al*., 1991), three originated in Middle America (races Durango, Jalisco, and Mesoamérica) and three in Andean South America (races Chile, Nueva Granada, and Perú).

P. vulgaris is diploid, with 11 chromosomes (2n = 22) and a relatively small genome (650 Mbp). In contrast of other legumes of the tribe Phaseolae such as soybean, the level of duplications and the amount of highly repeated sequences are low. Most loci are single copy and gene families tend to be small (Vallejos *et al*., 1992; Freyre *et al*., 1998; McClean *et al*., 2002).

3. Biological nitrogen fixation

Generally, plants acquire the nitrogen from the available sources in the soil, however, when this nutrient is scarce, some plants are able to obtain it from the atmosphere through the symbiotic relationship with bacteria capable to reduce the atmospheric N_2. Biological nitrogen fixation (BNF) is found in various plant families but is most prevalent in the Leguminosae (Sprent, 2002), which makes them very relevant for agricultural systems. Only cultivated legumes contribute with at least 70 million of tones of nitrogen per year (Brockwell *et al*., 1995). Soil bacteria, collectively called rhizobia, symbiotically interact with legume roots to form specialized structures called nodules. In most cases, the interaction is specific between one or a few species of rhizobia and the host plant. Common

Solamente las leguminosas cultivadas fijan anualmente alrededor de 70 millones de toneladas de N_2 atmosférico (Brockwell *et al*., 1995). De la interacción entre la raíz y el rizobio surgen unas estructuras especializadas denominadas nódulos. En la mayoría de los casos, la interacción es específica entre una especie de rizobio y una especie de planta hospedadora. En general, las leguminosas del género *Phaseolus* se encuentran formando simbiosis con diversas bacterias del género *Rhizobium*, como *R. leguminosarum* bv *phaseoli* (Dangeard, 1926), *R. etli*, (Segovia *et al*., 1993), *R. gallicum* (Amarger*et al*., 1997) y *R. tropici* (Martínez-Romero *et al*., 1991).

En las células infectadas de los nódulos, el rizobio se diferencia en una forma capaz de fijar el N_2 atmosférico, denominada bacteroide. La reducción del N_2 atmosférico se lleva a cabo por el complejo enzimático nitrogenasa de la bacteria, que cataliza la siguiente reacción: $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$. La reacción requiere una gran cantidad de poder reductor, que se genera a través de la respiración bacteriana. Por su parte, la planta suministra ácidos carboxílicos, principalmente malato, como fuente de energía para la bacteria (Lodwig y Poole, 2003).

Los nódulos de las leguminosas pueden ser de dos tipos: indeterminados o determinados. Los nódulos indeterminados son típicos de las leguminosas de climas templados, como el guisante y las especies del género *Medicago*. Estos nódulos mantienen una región meristemática en un extremo, lo que hace que tengan forma de varilla. Las leguminosas de climas tropicales o subtropicales, como la soja y la judía; y algunas de climas templados, como *Lotus japonicus*, desarrollan nódulos determinados, que no mantienen la región meristemática y tienen forma esférica (Sprent, 2008).

4. Leguminosas amídicas y ureídicas

El nitrógeno fijado se libera en forma de amonio al citosol de las células infectadas, donde es asimilado rápidamente a glutamina (Gln) por la glutamina sintetasa (Morey *et al*., 2002) (Fig. 1). Generalmente, la glutamina no se utiliza

bean is able to assimilate the atmospheric N_2 by establishing specific symbiotic relationships with soil N_2-fixing species of the genus *Rhizobium*, such as *Rhizobium leguminosarum* bv *phaseoli* (Dangeard, 1926), *Rhizobium etli*, (Segovia *et al*., 1993), *Rhizobium gallicum* (Amarger *et al*., 1997) and *Rhizobium tropici* (Martínez-Romero *et al*., 1991).

In the infected cells of nodules, rhizobia differentiate into bacteroids, a form able to fix nitrogen. The reduction of the atmospheric N_2 to ammonium is mediated by the bacterial complex nitrogenase which catalyzes the reaction: $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$. The reaction requires a large energy amount, generated through the bacteroid respiration, which is supported by reduced carbon, mainly as malate, supplied by the plant (Lodwig and Poole, 2003).

Legume nodules can be indeterminate or determinate. Indeterminate nodules maintain a meristematic region at the growing tip and are rod-shape. These nodules are developed mainly on the roots of temperate legumes such as pea and *Medicago* sp. On the other hand, legumes from tropical and subtropical regions like soybean and common bean, and some from temperate regions like *Lotus. japonicus* develop determinate nodules, which do not maintain a meristematic zone and are sphericals (Sprent, 2008).

4. Amidic and ureidic legumes

Fixed nitrogen as ammonium is released from the bacteroid to the cytosol of infected nodule cells and incorporated into the amide position of glutamine (Gln) in a reaction catalyzed by the enzyme glutamine synthetase (Morey *et al*., 2002) (Fig. 1). Generally, glutamine is a minor exported solute of nitrogen, which should be further transformed to other nitrogenous organic compounds used for the long-distance translocation from the site of fixation in nodules to the sites of utilization (Atkins, 1987). Legumes differ in the nitrogenous compounds that they export from the nodule to the shoot. Legumes originated from temperate zones export the amide asparagine as the main form of reduced nitrogen, whereas

como compuesto de transporte de nitrógeno y es transformada en otros compuestos orgánicos nitrogenados, que se translocan hacía los sitios de utilización (Atkins, 1987). Las leguminosas pueden transportar el nitrógeno fijado en los nódulos en forma de amidas (leguminosas amídicas) o en forma de ureidos (leguminosas ureídicas) (Atkins, 1991). Las leguminosas amídicas, como el guisante, Lotus y Medicago, exportan el nitrógeno fijado en forma de asparragina y son típicas de zonas templadas, mientras que las leguminosas ureídicas, como la soja y la judía, típicas de zonas tropicales, utilizan los ureidos alantoína y alantoato (Atkins, 1991), que son productos del catabolismo de las purinas.

El nitrógeno es un nutriente escaso para las plantas, que a menudo limita su crecimiento y reproducción. A diferencia de los animales, que utilizan los productos del catabolismo de las purinas como moléculas de desecho para eliminar el exceso de nitrógeno, las plantas son capaces de llevar a cabo la degradación completa de las purinas, lo que les permite recuperar todo el nitrógeno que contienen. Hasta el momento, no se conoce por qué algunas especies de leguminosas y no otras utilizan el catabolismo de las purinas para asimilar y exportar el nitrógeno fijado en sus nódulos.

La síntesis de ureidos conlleva un ahorro significativo de carbono. La cantidad de moléculas de carbono necesarias para sintetizar alantoína y alantoato es de 1 por cada molécula de nitrógeno, un valor muy bajo si se compara con el necesario para la síntesis de asparragina y glutamina, que es 2 y 2,5, respectivamente (Atkins y Smith, 2000). Además, aunque la síntesis de ureidos requiere la participación de 20 enzimas diferentes y la de asparragina solamente necesita cuatro pasos enzimáticos, el coste energético requerido para asimilar una molécula de nitrógeno en forma de alantoína es similar al requerido en la síntesis de asparragina (Smith y Atkins, 2002). Sin embargo, los ureidos son moléculas muy poco solubles en agua y se ha propuesto que su síntesis puede haberse visto favorecida solo en las leguminosas tropicales, para las que la disponibilidad de agua no es una limitación (Sprent y Embrapa, 1980; Schubert y Boland, 1990). El hecho de que la mayoría de las leguminosas tropicales tengan nódulos

legumes from tropical and subtropical zones use the ureides, allantoin and allantoate (Atkins, 1991), which are products of purine bases catabolism.

Nitrogen is a mineral nutrient scarce in many soils, therefore, plant growth is limited by its availability. Unlike animals, which use the products of purine catabolism as waste molecules to get rid of excess nitrogen, plants have evolved several enzymes for the complete degradation of purine bases, which allow them the recovery of the whole nitrogen contained in these compounds. Up to now, it remains largely unknown why some legume species and not others use this pathway to assimilate and export the nitrogen fixed in their nodules.

The synthesis of ureides allows a significant saving of carbon for ureidic legumes. The carbon molecules required for the assimilation of one molecule of nitrogen as allantoin and allantoate is 1, a low value compared with the required for the synthesis of asparagine and glutamine, 2 and 2.5, respectively (Atkins and Smith, 2000). Moreover, although the synthesis of ureides is carried out by 20 separate enzymes as compared with just four required for asparagine synthesis, the ATP and reducing equivalents cost needed to assimilate one molecule of fixed N_2 as ureides is not much different from that required for assimilate it as asparagine (Smith and Atkins, 2002). On the other hand, ureides are molecules with low water solubility and it has been hypothesized that their synthesis may have been favored only in tropical legumes in which water availability is not a common restriction (Sprent and Embrapa, 1980; Schubert and Boland, 1990). The fact that the tropical legumes all have determinate nodules suggests a relationship between the nodule type and the compounds for nitrogen translocation. However, there are some legumes bearing determinate nodules like *L. japonicus* that employ asparagine as a major translocation compound (Tajima *et al*., 2000). The same occurs with some temperate legumes with indeterminate nodules, suggesting that ureide accumulation in nodules of legumes would be due to some metabolic conditions in common between these species and not absolutely related to the type of nodule (Smith and Atkins, 2002).

determinados parece indicar que existe una relación entre el tipo de nódulo y los compuestos utilizados para la translocación del nitrógeno. Sin embargo, existen leguminosas amídicas como *L. japonicus* que también desarrollan nódulos determinados (Tajima *et al*., 2000) y leguminosas con nódulos indeterminados que transportan ureidos, además de asparragina. Este hecho sugiere que la síntesis de ureidos en los nódulos de las leguminosas no está directamente relacionada con el tipo de nódulo, sino con características metabólicas en común entre algunas especies (Smith y Atkins, 2002).

5. Síntesis de ureidos a partir del nitrógeno fijado

Para sintetizar ureidos a partir del N_2 atmosférico fijado en los nódulos de las leguminosas ureídicas se requiere la síntesis *de novo* de nucleótidos de purina y su posterior degradación hasta liberar las bases púricas. Posteriormente, las purinas serán oxidadas enzimáticamente hasta formar ureidos (Schubert, 1986; Atkins y Smith, 2000; Zrenner *et al*., 2006) (Fig. 1).
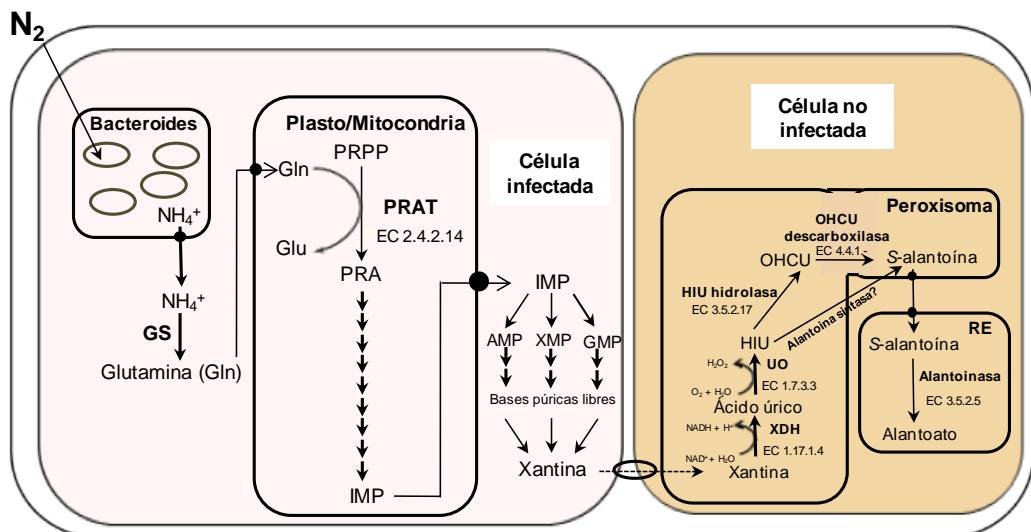
Fig. 1. Síntesis de ureidos a partir del N_2 fijado en los nódulos de las leguminosas ureídicas.

En los nódulos, la síntesis *de novo* de purinas tiene lugar en los plastos y las mitocondrias de las células infectadas. La primera reacción es llevada a cabo por la glutamina fosforribosilpirofosfato amidotransferasa (PRAT, EC 2.4.2.14) que

5. Ureide synthesis from fixed nitrogen

Synthesis of ureides from fixed N_2 requires the *de novo* synthesis of purine nucleotides and its further degradation to release purine bases, which are then enzymatically oxidized to form ureides (Schubert, 1986; Atkins and Smith, 2000; Zrenner *et al*., 2006) (Fig. 1).
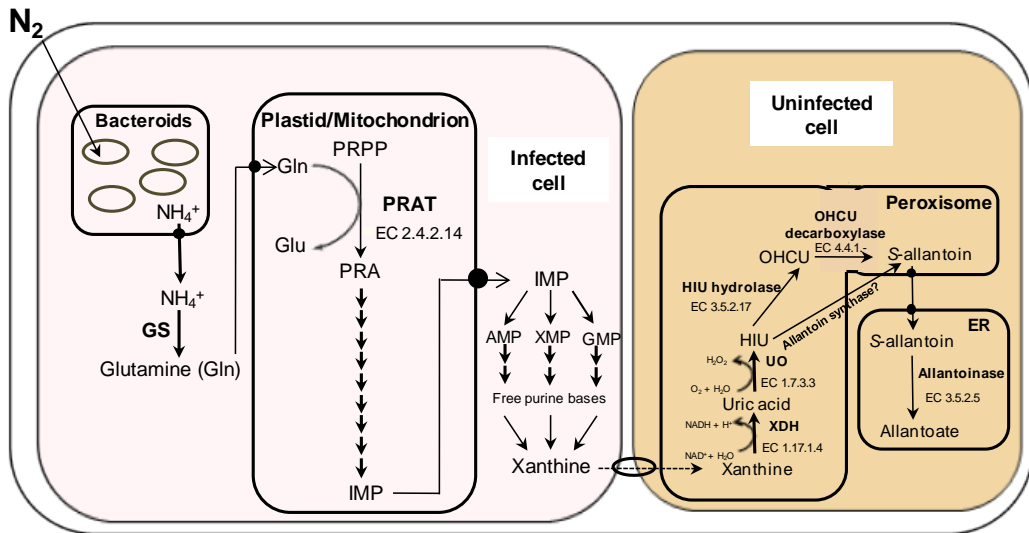
Figure 1. Synthesis of ureides from fixed N_2 in nodules of ureidic legumes.

In nodules, the *de novo* synthesis of purine nucleotides takes place in plastids and mitochondria of the infected cells. The first reaction is catalyzed by glutamine phosphoribosylpyrophosphate amidotransferase (PRAT, EC 2.4.2.14). This enzyme catalyzes the formation of phosphorybosylamine (PRA) from the glutamine imported from the cytosol and from the activated ribose precursor phosphoribosylpyrophosphate (PRPP) (Zrenner *et al*., 2006). PRAT catalyzes a key step in the control of *de novo* purine synthesis, since the enzyme is subjected to feedback inhibition by the end products nucleotides and is activated by its substrate glutamine (Reynolds *et al*., 1984; Kim *et al*., 1995). The following nine enzymatic steps lead to the synthesis of the first nucleotide, inosine monophosphate (IMP). Then, IMP serves as precursor for the synthesis of the other purine nucleotides, adenosine monophosphate (AMP), xanthosine monophosphate (XMP) and guanosine monophosphate (GMP). The degradation

cataliza la formación de fosforribosilamina (PRA) a partir de fosforribosilpirofosfato (PRPP) y de la glutamina importada desde el citosol (Zrenner *et al*., 2006). Este paso es clave para el control de la síntesis *de novo* de purinas, ya que la PRAT está sujeta a regulación por los productos finales de la ruta, los nucleótidos, y por su sustrato, la glutamina (Reynolds *et al*., 1984; Kim *et al*., 1995). Los nueve pasos enzimáticos siguientes dan lugar a la síntesis del primer nucleótidos purínico, inosina monofosfato (IMP), que sirve como precursor para la síntesis del resto de nucleótidos purínicos: adenosina monofosfato (AMP), xantosina monofosfato (XMP) y guanosina monofosfato (GMP). La degradación de los nucleótidos de purina para dar lugar a purinas libres se lleva a cabo por la desfosforilación de los nucleótidos y la hidrólisis de los correspondientes nucleósidos, reacciones catalizadas por enzimas con actividad nucleotidasa y nucleosidasa, respectivamente. En los nódulos de las leguminosas ureídicas, la degradación de los nucleótidos de purina converge en la base púrica xantina (Zrenner *et al*., 2006), que es transportada simplásticamente hacia las células no infectadas del nódulo, donde se transforma en ácido úrico por la enzima xantina deshidrogenasa (XDH, EC 1.17.1.4) (Triplett *et al*., 1980; Nguyen *et al*., 1986; Datta *et al*., 1991). A continuación, el ácido úrico es oxidado a 5-hidroxiisourato (HIU) por la enzima urato oxidasa (uricasa, UO; EC 1.7.3.3) (Kahn *et al*., 1997), localizada en el peroxisoma (Hanks *et al*., 1981). Para la síntesis de alantoína, el HIU es hidrolizado a 2-oxo-4-hidroxi-4-carboxi-5-ureidoimidazolina (OHCU) por la enzima HIU hidrolasa (EC 3.5.2.17) y éste es transformado en *S*-alantoína por la enzima OHCU descarboxilasa (EC 4.1.1.-). Curiosamente, en *Arabidopsis thaliana* se ha identificado una enzima capaz de catalizar ambas actividades denominada alantoína sintasa (Ramazzina *et al*., 2006) y, en soja, han sido identificadas al menos dos enzimas capaces de catalizar la hidrólisis del HIU: una HIU hidrolasa específica del nódulo y el dominio HIU hidrolasa de la alantoína sintasa (Sarma *et al*., 1999; Raychaudhuri y Tipton, 2002). Asimismo, todavía no se conoce si el OHCU puede ser también degradado por una enzima alternative o si el dominio OHCU descarboxilasa de la alantoína

of purine nucleotides to release free purine bases takes place through the nucleotide dephosphorylation and the hydrolysis of the corresponding nucleosides, reactions catalyzed by nuclease and nucleosidase enzymes, respectively. In the nodules of ureidic legumes all these pathways converge on xanthine, which is the central metabolite in purine nucleotide catabolism (Zrenner *et al*., 2006). Xanthine is transported symplastically to adjacent uninfected cells where it is oxidized to uric acid by the pivotal enzyme xanthine dehydrogenase (XDH, EC 1.17.1.4) (Triplett *et al*., 1980; Nguyen *et al*., 1986; Datta *et al*., 1991). Thereafter, uric acid is oxidized by the enzyme urate oxidase (uricase, UO; EC 1.7.3.3) (Kahn *et al*., 1997), located in peroxisomes (Hanks *et al*., 1981), to the intermediate 5-hydroxyisourate (HIU) (Kahn *et al*., 1997). For the synthesis of allantoin, HIU is hydrolysed by the enzyme HIU hydrolase (EC 3.5.2.17) to give 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) and OHCU is decarboxylated by the enzyme OHCU decarboxylase (EC 4.1.1.-) to give *S*-allantoin. Interestingly, a single polypeptide catalyzing both activities, called allantoin synthase, has been identified in *Arabidopsis thaliana* and in other plants (Ramazzina *et al*., 2006). In soybean, at least two enzymes able to catalyze the hydrolysis of HIU have been found: a nodule specific HIU hydrolase and the HIU hydrolase domain of the allantoin synthase (Sarma *et al*., 1999; Raychaudhuri and Tipton, 2002). Likewise, it is still unknown whether in soybean nodules OHCU is also degraded by an alternative enzyme or if the OHCU decarboxylase from allantoin synthase performs this function. The further oxidation of allantoin to allantoate is catalyzed by the enzyme allantoinase (EC 3.5.2.5) in the endoplasmic reticulum (ER) (Hanks *et al*., 1981; Werner *et al*., 2008) (Fig. 1).

6. Long distance transport of ureides

Once synthesized, allantoin and allantoate are translocated to the xylem and transported through the transpiration flow from the nodules to the source leaves (Atkins and Smith, 2000). Ureides can be also transiently stored in petioles or stems (Herridge *et al*., 1978) or may travel directly to sink tissues such as root

sintasa lleva a cabo esa función. La hidrólisis de la alantoína para dar lugar a alantoato es catalizada por la enzima alantoína amidohidrolasa (alantoinasa, EC 3.5.2.5) en el retículo endoplásmico (RE) (Hanks *et al*., 1981; Werner *et al*., 2008) (Fig. 1).

6. Transporte a larga distancia de los ureidos

La alantoína y el alantoato sintetizados en los nódulos a partir del N_2 atmosférico fijado son translocados al xilema y transportados desde los nódulos hacia las hojas fuente (Atkins y Smith, 2000) o almacenados transitoriamente en los peciolos y el tallo (Herridge *et al*., 1978). Además, parte de los ureidos pueden ser dirigidos directamente a los tejidos sumidero como los ápices de la raíz, flores, frutos y semillas (Layzell y Larue, 1982; Atkins y Beevers, 1990). Desde las hojas fuente, los ureidos son dirigidos por el floema hacia los tejidos sumidero, donde tiene lugar su degradación. El amonio liberado es utilizado para sintetizar aminoácidos, que constituyen las moléculas básicas para el crecimiento y el desarrollo de frutos y semillas (Winkler, 1987; Todd *et al*., 2006; Werner y Witte, 2011). Parte de los ureidos que llegan a las hojas son catabolizados, y el amonio liberado reasimilado en forma de aminoácidos, que pueden ser almacenados transitoriamente en forma de proteínas de reserva o utilizados en los procesos metabólicos de la hoja (Atkins, 1982; Costigan *et al*., 1987; Lansing y Franceschi, 2000; Todd *et al*., 2006).

7. Papel fisiológico de los ureidos

Las leguminosas ureídicas utilizan ureidos como compuestos de transporte de nitrógeno solamente cuando este procede de la fijación simbiótica (Matsumoto et al., 1977; Fujihara y Yamaguchi, 1980). Cuando la FBN es la única fuente de nitrógeno, la alantoína y el alantoato pueden llegar a representar el 85% del nitrógeno total transportado por el xilema. Sin embargo, si la planta crece en condiciones de fertilización con nitrato, el nitrógeno es exportado desde la raíz en forma de aminoácidos (principalmente asparragina y glutamina) y de nitrato, que

apices, sink leaves, flowers, fruits, and seeds (Layzell and Larue, 1982; Atkins and Beevers, 1990). In the source leaves, ureides are immediately loaded into the phloem for transport to sinks, where their complete catabolism takes place. Ammonium released is then re-assimilated by amino acid synthesis, which provides the building blocks for growth and development in sink tissues (Winkler 1987; Todd *et al*., 2006; Werner and Witte, 2011). Part of the ureides loaded in the source leaves are catabolized there and the released ammonium re-assimilated into amino acids, which are either employed in leaf metabolic processes or transiently stored in the form of vegetative storage proteins (Atkins, 1982; Costigan *et al*., 1987; Lansing and Franceschi, 2000; Todd *et al*., 2006).

7. Physiological role of ureides

The prevalence of the ureide export and the ureidic metabolism in tropical legumes occurs only when the main source of nitrogen is the symbiotic fixation in the nodules (Matsumoto *et al*., 1977; Fujihara and Yamaguchi, 1980). Ureide-producing legumes load different nitrogenated solutes into the xylem sap, depending of the form of nitrogenated nutrition. When BNF is the only nitrogen source, allantoin and allantoate may represent around 85% of the total nitrogen in the xylem sap. However, when these plants are fertilized with nitrate, amino acids (mainly asparagine and glutamine) and nitrate are the main forms of nitrogen exported from the roots (McClure and Israel, 1979; McClure *et al*., 1980). On the other hand, nitrate fertilization exerts a negative effect on nodule growth, nodule number and N_2-fixing activity, producing a variation in the xylem sap composition depending of the relative extents to which N_2 and nitrate are used (McClure and Israel, 1979; McClure *et al*., 1980; Pate *et al*., 1980). All these facts suggest a close association between N_2 fixation and ureide formation and, for a long time, it has been assumed that ureides reach significant concentrations only in nodulated, nitrogen-fixing plants.

Based in this fact, the determination of stem or petiole ureide levels was established as a reliable non-destructive method to estimate the nitrogen fixation

se almacena en las hojas (McClure y Israel, 1979; McClure *et al*., 1980). La fertilización con nitrato ejerce un efecto negativo en el crecimiento, el número y la actividad fijadora de los nódulos, dando lugar a variaciones en la composición nitrogenada del xilema, que dependen de la cantidad de nitrato utilizado (McClure y Israel, 1979; McClure *et al*., 1980; Pate *et al*., 1980; Díaz-Leal *et al*., 2012). Dada la estrecha relación entre la fijación de N_2 y la formación de ureidos, durante muchos años se ha asumido que los ureidos solamente alcanzan concentraciones significativas en las plantas que están llevando a cabo la fijación del N_2 atmosférico.

Basándose en este hecho, la determinación del contenido de ureidos en tallos y peciolos se estableció como un método reproducible y no destructivo para la estimación de la tasa de fijación de N_2 en las leguminosas ureídicas (McClure *et al*., 1980; Pate *et al*., 1980; Herridge, 1982; Patterson y Larue, 1983; Herridge y Peoples, 1990). Sin embargo, el nivel de ureidos en el xilema está fuertemente influido por el desarrollo de la planta, incrementando considerablemente durante la fase reproductiva y la senescencia (Schubert, 1981; Herridge y Peoples, 1990; Aveline *et al*., 1995; Díaz-Leal *et al*., 2012), momentos que coinciden con el inicio de la senescencia de los nódulos y, por tanto, con el cese de la fijación de N_2 (Díaz-Leal *et al*., 2012). Además, en algunas plantas no leguminosas también se han encontrado concentraciones altas de ureidos (Schubert y Boland, 1990; Schmidt y Stewart, 1998), lo que indica que los ureidos pueden ser generados a partir de fuentes distintas a la FBN.

La acumulación de ureidos también ocurre durante la germinación de las semillas (Quiles *et al*., 2009) y cuando las plantas son sometidas a estrés, situaciones en las que tiene lugar una alta removilización de nitrógeno (Hesberg *et al*., 2004; Brychkova *et al*., 2008; Alamillo *et al*., 2010). Por tanto, es probable que los ureidos que se sintetizan en esas condiciones procedan del reciclaje del nitrógeno liberado durante la removilización de las proteínas y/o los ácidos nucleicos. Además, se ha demostrado que los ureidos pueden también tener un papel protector frente al estrés oxidativo (Nakagawa *et al*., 2007; Brychkova *et al*.,

rates in nodules of tropical legumes (McClure *et al*., 1980; Pate *et al*., 1980; Herridge, 1982; Patterson and Larue, 1983; Herridge and Peoples, 1990). However, the level of ureides in xylem sap and in leaves of ureidic legumes is strongly influenced by the developmental stage of the plant, increasing both during the reproductive phase and the senescence (Schubert, 1981; Herridge and Peoples, 1990; Aveline *et al*., 1995; Diaz-Leal *et al*., 2012), stages in which nodules begin to senesce and loss their function (Díaz-Leal *et al*., 2012). Moreover, elevated concentrations of ureides have been found in amidic legumes (Atkins, 1991) and in several non-legume species (Schubert and Boland, 1990; Schmidt and Stewart, 1998) further indicating that ureides can be generated from sources others than BNF.

Ureide accumulation also occurs in situations such as germination (Quiles *et al*., 2009), natural senescence, darkness and abiotic stresses in which a high rate of nitrogen remobilization takes place (Hesberg *et al*., 2004; Brychkova *et al*., 2008; Alamillo *et al*., 2010), so it is likely that the enhanced ureides levels came from the recycling of nitrogen from proteins and/or nucleic acids already presents in the cells. In these situations, it has been shown that ureides can also act as scavengers of reactive oxygen species (ROS) (Nakagawa *et al*., 2007; Brychkova *et al*., 2008; Watanabe *et al*., 2010; Watanabe *et al*., 2014a) and participate in the signaling pathways that are triggered in the cell (Watanabe *et al*., 2014b). Therefore, it is likely that the ureides have a dual physiological role serving as efficient molecules for the remobilization and transport of nitrogen and acting as protectant from abiotic stresses.

8. Work hypothesis

In *P. vulgaris*, the synthesis of ureides is enhanced during water deficit conditions independently of N_2 fixation (Alamillo *et al*., 2010). However, the exact origin of these ureides remains unknown. The synthesis of ureides occurs through the oxidation of purines synthesized *de novo* or through oxidation of purines released during the catabolism of nucleotides already present in the cell (Zrenner *et al*.,

2008; Watanabe *et al*., 2010; Watanabe *et al*., 2014a) y participar en las rutas de señalización de respuesta al estrés que tienen lugar en la planta (Watanabe *et al*., 2014b). Por tanto, es probable que el papel fisiológico de los ureidos sea doble, constituyendo moléculas eficaces en el transporte y la remobilización de nitrógeno y sirviendo como protectores frente al estrés.

8. Hipótesis de trabajo

En *P. vulgaris*, la síntesis de ureidos incrementa en condiciones de estrés hídrico independientemente de la fijación de N_2 (Alamillo *et al*., 2010). Sin embargo, el origen de dichos ureidos no se conoce con exactitud. La síntesis de ureidos tiene lugar a partir de la oxidación de purinas sintetizadas *de novo* o procedentes del catabolismo de nucleótidos de purina preexistentes en la célula (Zrenner *et al*., 2006). En este trabajo se ha llevado a cabo la comparación y el análisis de la acumulación de ureidos y la sensibilidad a la sequía en diversos genotipos de judía. Asimismo, se ha pretendido dilucidar cuál es el origen de los ureidos acumulados durante la sequía a través de la caracterización y el análisis de la regulación de las enzimas glutamina fosforribosilpirofosfato amidotransferasa (PRAT) y xantina deshidrogenasa (XDH), que participan y controlan la síntesis *de novo* y la oxidación de las purinas, respectivamente. La hipótesis de trabajo es que si la acumulación de ureidos procede de la inducción de la síntesis *de novo* de purinas para aprovechar el carbono y el nitrógeno que se liberan tras la removilización de las moléculas orgánicas, la PRAT debería inducirse o activarse en las condiciones de estrés, al menos en las variedades más sensibles, que acumulan más ureidos. En cambio, si el origen de los ureidos es la degradación de los nucleótidos de purina preexistentes, sería solamente la vía de oxidación de las purinas y, por tanto, la XDH, la que se induciría en ésas condiciones. Por otra parte, tanto el análisis de las variaciones en la sensibilidad a la sequía y la acumulación de ureidos, como la caracterización molecular y bioquímica de los mecanismos involucrados en esas variaciones podrían ser de utilidad en futuros programas de mejora y aportarían información relevante sobre enzimas clave en

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2006). In this work, the comparison and the analysis of the accumulation of ureides and sensitivity to drought in different common bean genotypes has been carried out. Likewise, our aim was to dilucidate the actual origin of the ureides that accumulated during drought conditions, through the characterization and analysis of the enzymes glutamine phosphoribosylpyrophosphate amidotransferase (PRAT) and xanthine dehydrogenase (XDH), which participate and regulate the *de novo* synthesis and the oxidation of purines, repectively. The hypothesis of this work is that if the accumulation of ureides occurs as a result of the induction of the *de novo* synthesis of purines, recycling the carbon and nitrogen released after the remobilization of organic molecules, the activity of PRAT should be induced during water stress, at least in the most sensitive genotypes, which are those that accumulate more ureides. However, if the source of the ureides is the purine bases released during the degradation of preexisting nucleotides, only the XDH should be induced. On the other hand, the analysis of the variations in the sensitivity or tolerance to drought between genotypes, as well as the molecular and biochemical characterization of the mechanisms that control these variations, may be a useful tool in future breeding programs and would provide important information on key enzymes in the regulation of the synthesis of purines and ureides.

la regulación de la síntesis de purinas y ureidos.

9. Referencias

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OBJECTIVES

The general objective of this work has been to elucidate the origin of the ureides accumulated in the leaves from *Phaseolus vulgaris* when plants are exposed to water deficit.

With this purpose, the specific objectives were:

1. The comparison of ureide metabolism and biological N_2 fixation responses to drought stress between different genotypes of *P. vulgaris*.

2. Isolation and characterization of the gene coding for xanthine dehydrogenase in *P. vulgaris*.

3. Analysis of the expression and the activity of PvXDH during water stress and other stress-related treatments.

4. Identification, isolation and characterization of the genes coding for glutamine phosphoribosylpyrophosphate amidotransferase (PRAT) in *P. vulgaris*.

5. Heterologous expression and biochemical characterization of PvPRATs proteins.

6. Analysis of the regulation of PvPRATs under physiological and during stress conditions.

CHAPTER 2: COMPARISON OF N_2 FIXATION INHIBITION AND UREIDE ACCUMULATION UNDER WATER DEFICIT IN FOUR COMMON BEAN GENOTYPES OF CONTRASTING DROUGHT TOLERANCE

Abstract

Soil drought is the principal constraint of world production in legume crops. There is a considerable variability of N_2 fixation sensitivity to drought among genotypes that has been related with accumulation of ureides in soybean. Our aim was to search for genotypic differences in drought sensitivity and ureide accumulation in common bean germplasm. Changes in response to water deficit of the N_2 fixation rates, ureide contents and the expression and activity of key enzymes for ureide metabolism were measured in four common bean genotypes showing variable drought tolerance. We found a variable degree of drought-induced N_2 fixation inhibition among common bean genotypes. Together to the N_2 fixation inhibition there was accumulation of ureides in stems and leaves of sensitive and tolerant genotypes, although it was higher in the leaves of the most sensitive ones. In contrast, there was no accumulation of ureides in the nodules or roots from stressed plants. In addition, level of ureides in the most sensitive genotype rises after N_2 fixation inhibition. The results indicated that shoot ureide accumulation after long exposure to drought could not be the actual cause of N_2 fixation inhibition as suggested in soybean. In contrast, ureides seem to be produced as part of a general response to stress, and therefore higher accumulation might correspond to a higher sensitivity to the stressful conditions.

Introduction

Common bean (*Phaseolus vulgaris* L.) is the main food protein crop for direct consumption worldwide. It is a staple food in developing countries, where beans are main dietary supply of plant proteins (Graham and Ranalli, 1997; Broughton *et al.*, 2003; Graham and Vance, 2003). Beans, as other legumes, are able to use atmospheric N_2 through the symbiotic associations with soil N_2-fixing rhizobia. This capacity is one of the main advantages of this crop because it reduces the nitrogen fertilizers demands with a positive impact in the economy, especially in developing countries, and in the environment, contributing to the reduction of the emission of greenhouse gas (NOx). Among the several constraints that affect legume crops (Zharam, 1999) drought is the principal factor limiting common bean yield. In particular, the biological nitrogen fixation (BNF) in the symbiotic system bean-rhizobia is strongly inhibited under water deficit, so that the amount of nitrogen compounds required for biomass and seed production are seriously compromised under water-stress conditions (Sinclair and Serraj, 1995; Serraj *et al.*, 1999; Serraj, 2003; Purcell *et al.*, 2004; Arrese-Igor *et al.*, 2011). The existence of genetic variation in N_2 fixation response to abiotic stresses among legume genotypes opens the possibility for enhancing N_2 fixation tolerance through selection and breeding.

Ureides (allantoin and allantoate) are the main nitrogen-rich molecules used by ureidic legumes, as soybean (*Glycine max* L.) and common bean, to transport the nitrogen fixed in the nodules to the whole plant tissues (Kohl et al., 1999; Boldt and Zrenner, 2003; Zrenner *et al*., 2006). In ureidic legumes, ureides are formed through the oxidative catabolism of the purines synthesized *de novo* in the nodules, incorporating the recently fixed nitrogen. Among the enzymatic steps leading to the synthesis of ureides, urate oxidase (uricase, UO; EC 1.7.3.3), catalyzing the oxidation of uric acid, has a key role in nodule development and funtion (Bergman *et al*., 1983; Le *et al*., 1993). The direct product of uricase, 5-hydroxyisourate (HIU), is probably transformed by the bifunctional enzyme

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allantoin synthase (Lamberto *et al*., 20010; Pessoa *et al.*, 2010) to allantoin, which is then oxidized to allantoate by allantoin amidohydrolase (allantoinase, ALN; EC 3.5.2.5) (Webb and Lindell, 1993; Raso e*t al.,* 2007). ALN is a key enzyme in the metabolism of ureides, catalyzing the first step in the degradation of the ureide allantoin and the synthesis of allantoate, the second most prominent ureide. Allantoate amidohydrolase (AAH; EC 3.5.3.9) catalyzes then the breakdown of allantoate to the unstable ureidoglycine compound (Winkler *et al*. 1985; 1987; Werner *et al.,* 2008; 2010; Serventi *et al.,* 2010). At least two more enzymatic reactions are needed to yield glyoxylate, the last product of ureides degradation, releasing the four nitrogen atoms from the ureide molecule (Werner *et al.,* 2013).

In soybean, several works have shown that differences in the N_2-fixation tolerance to drought among different genotypes are inversely correlated to plant ureide concentrations under water deficit conditions (Serraj and Sinclair, 1996; Purcell *et al*., 2000; Sinclair *et al.*, 2000; Charlson *et al.*, 2009; Gil-Quintana *et al*., 2013). The accumulation of ureides in shoots of susceptible soybean genotypes under water deficit has been shown in various reports, supporting the idea that high ureide levels are likely associated with N_2 fixation inhibition (Purcell *et al.*, 2000; Vadez and Sinclair, 2001; King and Purcell, 2005; Charlson *et al*., 2009). Moreover, Ladrera *et al.* (2007) also proposed ureide accumulation as related to N_2 fixation inhibition, but, in contrast to previous reports, accumulation of ureides was only observed in the nodules and not in aerial tissues of the drought-stressed soybeans.

In addition, it has been proposed that ureide exporting legumes are more drought-sensitive than the amide exporting ones (Sinclair and Serraj, 1995). Thus, a N-feedback regulation hypothesis, in which ureides would be among the signalling molecules triggering the inhibition, has been proposed to explain the reduction of N_2 fixation under drought stress conditions in soybean (Serraj *et al*., 1999; King and Purcell, 2005), although the actual mechanism involved in the inhibition remains unclear. The differential ureide accumulation between tolerant and

sensitive genotypes has been associated with the inhibition of the Mn-dependent enzyme AAH in the most sensitive ones (Vadez and Sinclair, 2001; Sinclair *et al.*, 2003). Hovever, the expression of AAH did not change under drought conditions (Charlson *et al.*, 2009), or even was slightly stimulated (Alamillo *et al*, 2010), and a postranscriptional inhibition of AAH protein has been proposed as the possible cause of allantoate accumulation.

In the genetic pools (Andean and Mesoamerican) of common bean there is a wide variability in phenotipyc characters, including morpho-agronomic traits (Singh *et al.,* 1991; Escribano *et al.*, 1994; Casquero *et al*., 2006; González *et al.*, 2006;), stress tolerance and ability to make an effective symbiotic association with rhizobia (Rodiño *et al.* 2011). In contrast to the intensive search in soybean, there have been only a few studies on genotypic diversity of common bean N_2 fixation tolerance to drought (Riveiro, 2012), and its possible relationship to the low accumulation of ureides. In an early work, Serraj and Sinclair (1998) showed variation in ureide concentrations despite the lack of significant differences in the N_2 fixation response to drought in two genotypes of common bean. Genetic variability in N_2 fixation resistance to drought has been further corroborated in a comparison of 12 common bean genotypes by Devi *et al*. (2013). However, the possible correlation of the resistance to the concentration of ureides was not examined in this study.

In a common bean breeding line belonging to the Great Northern market class (Santalla *et al.*, 2001), we recently showed that prolonged periods of water deficit led to the progressive accumulation of the ureide allantoate in roots, stem and leaves but not in nodules of plants subjected to drought (Alamillo *et al.*, 2010). Moreover, this accumulation was attributed to the induction of ALN activity, which catalyzes the synthesis of allantoate, and not to a decrease in the degradation of allantoate. Moreover, it was found that allantoate increased even when N_2 fixation was nearly or completely inhibited by drought, as well as in non-nodulated plants, therefore indicating that these ureides could not be the result of N_2 fixation held in nodules, neither the cause of its inhibition, but

instead, the drought should have induced alternative routes of ureide synthesis in aerial tissues (Alamillo *et al.*, 2010).

The aim of this work was to analyze ureide metabolism in two breeding lines of common bean, reported to have differences in their tolerance to soil water deficit (Frahm *et al.*, 2003) and two landraces that showed differential yields under elevated or restricted rainfall conditions (Riveiro, 2012). For that, we have determined whether there are genotypic differences in the accumulation of ureides in common bean germplasm with different sensitivity of N_2 fixation to drought. Moreover we have measured the levels of gene expression and enzyme activities of the key ureide metabolism enzymes, ALN and AAH, to further determine the actual cause of ureide accumulation under these conditions.

Materials and methods

1. Plant material, growth conditions and drought treatment

The studied common bean genotypes were the landraces PHA-0246 and PHA-0683 and the breeding lines PMB-0285 and PMB-0306 (Table 1 and Supplementary Fig. S1), form the germplasm collection at the MBG-CSIC (Pontevedra, Spain) (De Ron *et* al., 1997).

Table 1. Origin, market class, seed colour and size of the common bean genotypes used in this study.

GENOTYPE NAME	GENETIC MATERIAL	ORIGIN	MARKET CLASS	SEED COLOUR	SEED SIZE (g.100seeds^{-1})
PHA-0246	Landrace	Pontevedra, Spain	Canellini	White	35
PHA-0683	Landrace	Monção, Portugal	Mottled canellini	Brown, red stripped	38
PMB-0285 [1]	Breeding line	University of Michigan	Black turtle	Black	27
PMB-0306 [2]	Breeding line	University of Idaho	Brown garbanzo	Light brown	29

[1] Provided by Prof J. D. Kelly in 2005 as L88-18. Drought sensitive
[2] Provided by Prof S. P. Singh in 2005 as SEA 5. Drought tolerant

The seeds were maintained in cold storage rooms at 4 ℃ and 40% of relative humidity. *Rhizobium leguminosarum* ISP 14 (HUP[-]) strain was provided by CIFA-Las Torres (Sevilla, Spain). Seeds were surface-sterilized by sequential dipping in ethanol (30 s) and 0.2% (w/v) sodium hypochlorite (5 min) and then washed thoroughly with distilled water. Soaked seeds were allowed to germinate in Petri dishes (120 mm diameter) with wet paper under sterile conditions. After germination, 4 seedlings were sown on each pot (16 cm diameter, 18 cm height) filled with an artificial substrate composed of vermiculite/perlite mixture (2/1 w/w) and inoculated with a fresh suspension of *Rhizobium leguminosarum* ISP 14, which had been cultured at low temperature (28 °C) for less than 30 hours. Plants were cultured in growth chamber under a long-day photoperiod (16 hours light, 8 hours dark), 300 $\mu E.m^{-2}.s^{-1}$ lighting, and 70% relative humidity at 26-21 °C (day-night temperatures). Inoculated plants were watered three times a week with nitrogen-free nutrient solution (Rigaud and Puppo, 1975).

Drought stress treatments started at the onset of flowering, which happened at 25–28 days after sowing, according to bolting dates of each genotype. Plants were randomly separated into two sets and watering was withheld to one half of the plants, whereas the second half was regularly irrigated. N_2 fixation measurement and plant collection was done at 0 (before drought treatment), 7 and 14 days of drought treatment. Soil water capacity (SWC) was gravimetrically determined, as described in Charlson *et al.* (2009). Basically, pots filled with substrate were watered in excess, overnight let to drain and weighed to estimate the 100% SWC at sowing. Weighing of pots was repeated during the drought treatments for both, control and drought stressed plants. SWC was maintained at about 80-90% for control plants. For water-stressed plants, the SWC at sampling time ranged from 50-55% at 7 days and was around 30% after 14 days of water withholding as reported previously (Alamillo *et al.*, 2010).

For biomass estimation, FW and DW of the control and treated plants were determined at the sampling times. Tissue samples were weighed fresh and desiccated in an oven at 72 ℃ during 48-72 hours before dry weight

determination.

Relative water content (RWC) of leaves was measured according to Barrs and Weatherly (1962). The leaves were weighed immediately after sampling and soaked overnight in distilled water at 4 ºC. After the incubation, the leaves were blotted dry and weighed prior to drying at 80 ºC for 24 hours. Subsequently, dry weight of the leaves was determined. The leaf RWC was calculated using the following formula: RWC = ((FW − DW)/(TW-DW)) x 100, where FW is fresh weight, DW is dry weight, and TW is turgid weight considered as the weight after the leaf was kept overnight in distilled water.

Plant material collected at the indicated times was frozen with liquid nitrogen and stored at -80 ºC until further analysis. As a routine, the following samples were collected: fourth trifoliate leaves; shoot tissue portions including basal, medium and apical stem after removal of leaves; whole roots collected after carefully removal of nodules. Nodules collected from each individual plant were weighed and photographed, as a visual control of N_2 fixation capacity (Supplementary Fig. S2).

2. Nitrogen fixation measurement

Nitrogenase (EC 1.7.9.92) activity was measured as the representative H_2 evolution in an open-flow system (Witty and Minchin, 1998) using an electrochemical H_2 sensor (Qubit System Inc., Canada). For that, nodulated roots excised from their shoots just before the activity assay were sealed in 0.125 L cylinders and H_2 production was recorded according to the manufacturer´s instructions. Apparent nitrogenase activity (ANA, rate of H_2 generation in air) was determined under N_2:O_2 (80%:20%) with a total flow of 0.4 L.min^{-1}. After reaching steady-state conditions, total nitrogenase activity (TNA) was determined under Ar:O_2 (79%:21%). Standards of high-purity H_2 were used to calibrate the detector. Roots attached to whole plants were also measured to ascertain that the separation from shoots did not cause a significant reduction on nodule activity during the assay.

3. Gene expression analysis

Total RNA was isolated from the different tissues using the TRI REAGENT® (Sigma-Aldrich) following the manufacturer's instructions. Prior to RT-PCR, total RNA from control and treated tissues was incubated with RNAase-free DNAaseI (Promega, Madison WI, USA) at 37 ºC for 30 min to eliminate any traces of genomic DNA. Lack of amplification of the 18S rRNA was used to check the successful removal of DNA. First strand cDNA synthesis was done from 2.5 µg of DNAase-treated RNA using a iScript™ reverse transcriptase (Bio-Rad), following the manufacturer's instructions. Expression analysis was performed by quantitative RT-PCR (qRT-PCR) in an iCycler iQ System (Bio-Rad, Hercules, CA, USA) using the iQ SYBR-Green Supermix (Bio-Rad) and gene-specific primers (Table 2). The PCR programme consisted of an initial denaturation and Taq polymerase activation step of 5 min at 95 ºC, followed by 50 cycles of 15 s at 95 ºC and 1 min at 60 ºC. Expression of *Actin*-2 and 18S genes was used as internal controls, after checking that its level of expression was similar in all samples. The specificity of primers was verified using amplicon dissociation curves and by sequencing of the PCR products. The amplification efficiency of each primers pair, calculated by PCR using serial dilutions of root and leaf cDNAs, was >80%. The threshold cycle (C_T) values were in the range of 20–23 cycles for *Actin*-2 gene, 21–30 cycles for the genes of ureide-metabolism and 14-17 cycles for 18S rRNA. Analysis of relative gene expression was calculated from ΔC_T values (Pfaffl, 2001), using expression of *Actin-2* gene for data normalization. All the reactions were set up in triplicate (three technical replicates) using three to five RNA preparations (biological replicates) from different plants.

Table 2. Primers used for qRT-PCR.

Gene	Primers sequences (5´- 3´)
PvAct-2 (KF033666.1)	ACT2-D (GGAGAAGATTTGGCATCACACGTT)
	ACT2-R (GTTGGCCTTGGGATTGAGTGGT)
Pv18S (CV670768.1)	18S-D (GACACGGGGAGGTAGTGACAAT)
	18S-R (ACGAGCTTTTTAACTGCAACAACT)
PvALN	ALN-D (GTGGGAAGGATTTGATACTGGTAC)
	ALN-R (TAGACACGGTTGTAGGGTAATTGT)
PvAAH	AAH-D (GAGATTGGGAAGCATTGGTGCCTA)
	AAH-R (GTGGGGCGGCTGTTTATGGT)

4. Allantoinase and allantoate amidohydrolase enzymatic assays

Allantoin degrading activity was measured as described in Alamillo *et al.* (2010), following the production of allantoate as indicated by Raso *et al.* (2007). AAH activity was determined according to a modification of the assay described in Werner *et al.* (2008). All procedures for crude extract preparation were carried out at 0–4 °C. Frozen plant material was ground to a fine powder under liquid nitrogen. Plant extracts were obtained by adding 2 mL of extraction buffer per gram of tissue. The extraction buffer for AAH assay was 100 mM Tea-NaOH, pH 8; 150 mM NaCl; 15 mM DTT; 1 mM $MnCl_2$; 0.5% (w/v) Triton X-100 and 50 µM phenylphosphorodiamidate (PPD). The resulting homogenate was centrifuged at 15000 *g* for 10 min and the supernatant was dialyzed through *SpinTrap G25* columns previously equilibrated with wash buffer, and the flow-through was used as crude extract. The wash buffer was 100 mM Tea-NaOH, pH 8; 150 mM NaCl; 2 mM DTT; 2 mM $MnCl_2$; 0.005% (w/v) Triton X-100 and 100 µM PPD. The enzymatic assay was carried out at 37 ºC, in a reaction mixture composed of 50% (v/v) 6 mM allantoate solution in 10 mM Tea-NaOH, pH 8.0; 40% (v/v) wash buffer and 10% (v/v) of the previously dialyzed crude extract. Aliquots were taken at several time points and allantoate degrading activity was determined following the production of ammonium as indicated by Witte and

Medina-Escobar (2001). Soluble protein was measured in the same extracts in which the enzymatic activities from control and drought-stressed tissues were determined (Bradford, 1976).

One unit (U) of enzymatic activity is the amount of enzyme that catalyses the transformation of one μmol of substrate per min. Results of enzymatic activity are given as mU per mg soluble protein. The results are expressed as means of the values from at least three independent experiments. Enzymatic assays and analytical determinations from each biological experiment were done at least twice (two independent samples) and each assay was measured by duplicate (technical replicates).

5. Ureide determination

The concentration of ureides was determined by the colorimetric assay of glyoxylate derivatives (Vogels and Van der Drift, 1970). In this method, allantoin and allantoate are independently determined after their chemical transformation to glyoxylate. The values of total ureides in crude extracts correspond to the sum of allantoin plus allantoate. For each assay, 100 mg of plant tissue were homogenized in 0.4 mL of 50 mM Tris–HCl, pH 7.8; 100 mM $MgSO_4$ and 0.15% (w/v) deoxycholic acid (DOC) and centrifuged at 15000 g for 10 min to remove cell debris. The supernatant was immediately used to estimate the ureide content. For each biological experiment, determinations were done by duplicate (technical replicate).

6. Experimental design and statistical analysis of the data

Plants cultured under well irrigated conditions were randomly distributed into control and treated groups and the measurements were conducted at 0, 7 and 14 days of drought treatment. The experiment was repeated two times, and 10-12 plants per treatment were analyzed in each sampling time. N_2 fixation was measured individually in each plant, while tissues from several independent

plants were pooled together for the determination of ureide concentration.

The experiments were designed as a randomized block with two replications. Each replication consisted of 10-12 individuals per sampling time and treatment in N_2 fixation analysis and five individuals per sampling time in the case of ureide determination. *Student´s t*-test statistical analysis was performed using the *GraphPad* software package. ANOVA, Fisher's LSD test and the Pearson correlation matrix were computed using XLSTAT software, version 2011.4.02 (Addinsoft, 2011).

Results

1. Differential N_2 fixation inhibition

N_2 fixation was measured in plants of PHA-0246, PHA-0683, PMB-0285 and PMB-0306 genotypes at 0, 7 and 14 days of treatment both in control fully-irrigated and in drought-stressed plants. N_2 fixation was measured as the ANA according to Witty and Minchin (1998). As shown in Fig. 1, water deprivation caused a variable inhibition of N_2 fixation among the different genotypes, with lower inhibition of the N_2 fixation in PHA-0246 and PHA-0683 landraces than in the PMB-0285 and PMB-0306 breeding lines. After the first 7 days of water deficit, plants of PHA-0246 and PHA-0683 did not show a lower N_2 fixation rate than the corresponding controls, whereas plants of PMB-0285 and PMB-0306 showed a 54% and 23% inhibition, respectively. After 14 days of drought (severe stress) plants of PHA-0246 and PHA-0683 were still able to maintain around 50% of their N_2 fixation compared to control plants (Fig. 1). In contrast, N_2 fixation in PMB-0306 declined to about 20% of the well-irrigated controls and it was completely inhibited in PMB-0285 (Fig. 1). Statistical analysis of the data revealed that under well irrigated conditions, the four accessions behaved with no significant differences in their N_2 fixation rates. In contrast, the differences in N_2 fixation among the accessions were significant after 14 days of drought, with PHA-0683 and PHA-0246 showing higher performance under drought stress than

the lines PMB-0306 and PMB-0285 (Supplementary Tables S1, S2 and S3).
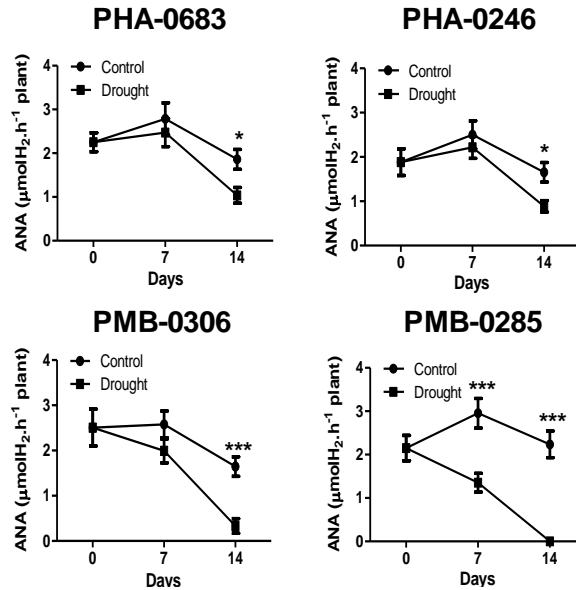
Fig. 1. Effect of drought stress on N$_2$ fixation of *P. vulgaris* landraces PHA-0683, PHA-0246 and breeding lines PMB-0306 and PMB-0285. N$_2$ fixation was represented as apparent nitrogenase activity (ANA) of plants well-watered (control) and drought-stressed (drought) before (0) and during the drought treatment (7 and 14 days). Genotypes are ordered according to their degree of tolerance. Data are mean of 2 independent experiments with 10-12 plants in each sampling time. Significant differences according to Student´s t-test are denoted by asterisks (* p <0.05, *** p<0.001).

Soil RWC was measured along the treatments. Results shown in Fig. 2A indicated that the decrease was similar for all four genotypes undergoing the water deficit treatment. Estimation of leaf RWC also showed that the leaves from all the genotypes were similarly affected by water deficit (Fig. 2B).
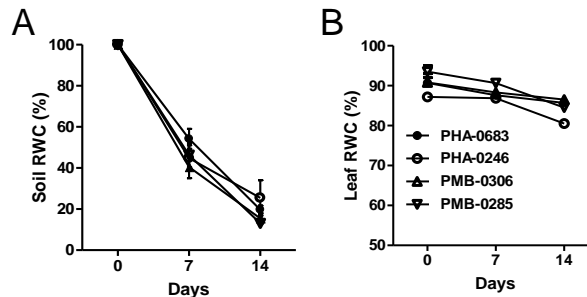
Fig. 2. Relative water content (RWC) after 7 and 14 days of water deficit. (A) RWC of soil. (B) RWC of leaves. Genotypes are ordered according to their degree of tolerance to N$_2$ fixation. Data are mean of 2 independent experiments with 10-12 plants in each sampling time.

Nodules from each genotype were differently affected by the stress. Nodules from PHA-0246 and PHA-0683 showed only a slight decrease in their dry mass in response to stress (18 and 38% reduction, respectively) and exhibited typical nodule senescence symptoms later than the nodules from the PMB-0285 and PMB-0306 plants. In contrast, water deficit caused a drastic reduction in PMB-0306 nodule dry weight (65%) and the complete lack of measurable nodules in PMB-0285 roots (Table 3 and Supplementary Fig. S2).

Table 3. Plant biomass changes in response to drought. Dry weight of the tissues from control and non-irrigated plants collected at 7 and 14 days of treatment. Significant differences between control and stressed samples are denoted by asterisks.

Genotype	Time (days)	Condition	Nodules (g DW)	Ratio	Roots (g DW)	Ratio	Shoots (g DW)	Ratio
PHA-0683	7	Control	0.027±0.002	1.20	0.168±0.024	1.70	1.700±0.130	0.85
		Drought	0.033±0.001		0.302±0.067		1.460±0.132	
	14	Control	0.039±0.003	0.82	0.241±0.052	2.30	2.020±0.220	0.91
		Drought	0.032±0.002		0.557±0.146		1.840±0.181	
PHA-0246	7	Control	0.030±0.006	1.03	0.123±0.025	1.84	1.700±0.120	0.95
		Drought	0.031±0.005		0.227±0.030		1.620±0.066	
	14	Control	0.051±0.004	0.62	0.250±0.077	1.45	1.790±0.170	0.81
		Drought	0.035±0.042		0.363±0.035		1.450±0.165	
PMB-0306	7	Control	0.030±0.005	1.20	0.253±0.073	1.25	0.860±0.162	1.07
		Drought	0.036±0.005		0.317±0.049		0.923±0.127	
	14	Control	0.031±0.004	0.35	0.300±0.030	1.84	1.850±0.270	0.77
		Drought	0.011±0.004*		0.552±0.040*		1.433±0.139	
PMB-0285	7	Control	0.029±0.006	0.87	0.218±0.014	1.53	0.602±0.053	1.00
		Drought	0.024±0.006		0.335±0.043		0.610±0.082	
	14	Control	0.047±0.007	0.00	0.212±0.040	2.40	1.081±0.070	0.76
		Drought	0*		0.510±0.100*		0.832±0.032*	

Roots and shoots biomass was also affected by the water deficit treatment, although the differences among genotypes were less pronounced than the effects observed in the nodules. In general, root biomass increased in response to the

stress in all genotypes, whereas shoot biomass suffered a 10% reduction in PHA-0683, 20% in PHA-0246 and around 25% in PMB-0285 and PMB-0306 plants (Table 3).

2. Ureides are accumulated in aerial tissues under drought conditions

To assess whether the N_2 fixation sensitivity of each genotype was related with the ureide content in plant tissues, the ureide concentration was determined in samples of nodules, roots, stems and leaves of plants from each genotype at 0, 7 and 14 days of drought treatment (Fig. 3). Tissues from controls (well irrigated plants) and non irrigated plants were collected and ureides measured at each sampling times.

The levels of ureides incremented in the stems and leaves of all four genotype with the drought treatment and the accumulation occurred mainly in the form of allantoate (Fig. 3C and 3D). However, the ureide levels in roots and nodules did not show any significant change during the experiment in any of the different genotypes (Fig. 3A and 3B). Neither after 7 days, nor after 14 days of drought, there was an increase in ureides in the stressed nodules, although the N_2 fixation was highly inhibited in the most sensitive, PMB-0285 and PMB-0306 breeding lines (Fig. 1 and 3A).

In contrast to the lack of effect on ureide concentration in roots and nodules, there was a significant accumulation of ureides under drought conditions in the stems of all the genotypes. Nevertheless, increases in ureide levels in stems did not show a higher increment with higher N_2 fixation sensitivity of the plants (Fig. 3C). In contrast, the leaves of the breeding lines PMB-0306 and PMB-0285, in which N_2 fixation was more inhibited by the treatment, accumulated more ureides that the more tolerant PHA-0246 and PHA-0683 landraces. Moreover, differences in the changes in leaf ureide levels among genotypes increased when the stress became more severe (Fig. 3D) Statistical analyses of the data corroborated that the ureide concentrations in the drought stressed leaves of PMB-0285 and PMB-0306 were significantly higher than in control plants (Supplementary Table S1),

and that there were significant differences in the accumulation of ureides among the genotypes (Supplementary Tables S2 and S3).
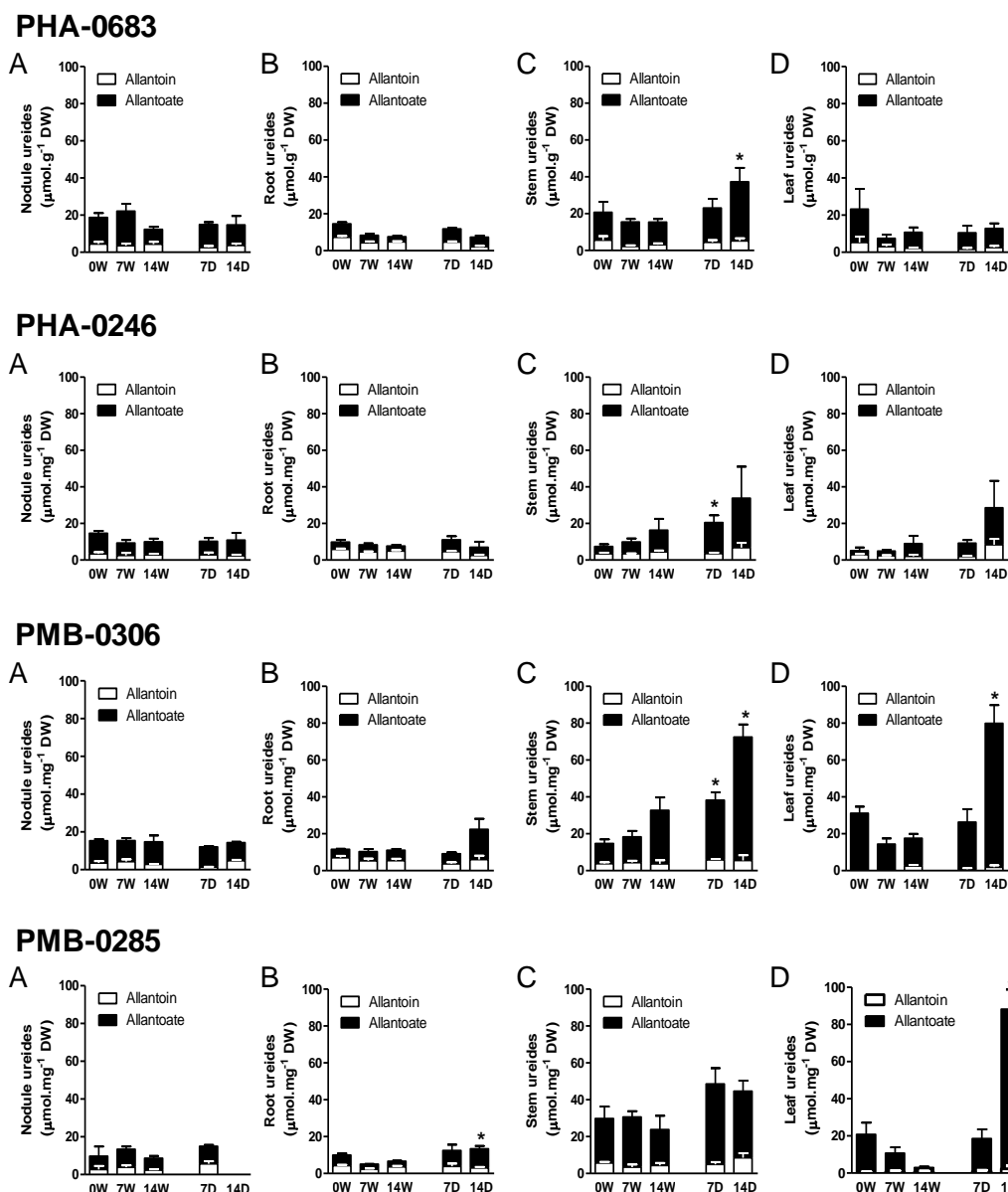
Fig. 3. Effect of drought stress on the ureide concentration in tissues from common bean genotypes. Allantoin and allantoate levels in (A) nodules, (B) roots, (C) stems and (D) trifoliate leaves from plants of PHA-0683, PHA-0246, PMB-0306 and PMB-0285 common bean genotypes well irrigated (W) and after drought stress (D) at 0, 7 and 14 days of treatment. Results are the mean of at least 5 measurements in each sampling time from two independent experiments. Significant differences according to Student´s t-test are denoted by asterisks (* $p<0.05$).

The differences between 7 and 14 days of drought were higher in the lines PMB-0306 and PMB-0285 than in the landraces PHA-0246 and PHA-0683 (Supplementary Tables S1, S2 and S3). Moreover, the Pearson´s scores for N_2 fixation and ureides, as differences between control and stressed plants, were significantly correlated, both at 7 and 14 days of drought (0.88** and 0.89**, respectively).

3. Changes in the expression of ureide metabolism genes in response to drought

To determine whether the rising values of tissue ureides (mainly allantoate) result from changes in the transcription of genes related to ureide metabolism in response to water stress, we used qRT-PCR to determine the mRNA levels of genes coding for the two key enzymes of allantoate metabolism: allantoinase (ALN) and allantoate amidohydrolase (AAH). The activity of ALN is responsible for the synthesis of allantoate from allantoin, whereas AAH catalyses allantoate degradation. Expression of these two genes was measured in nodules, stems and leaves from control, well-irrigated plants, and from plants subjected to drought stress during 7 days. The relative expression levels of the allantoate metabolism genes in tissues from drought stressed plants, compared with their expression in well-irrigated controls, are shown in Fig. 4.

Although there are two genes coding for ALN in common bean (Díaz-Leal *et al*., 2012), expression levels of one of them was negligible, and it is not shown in this figure. There were no major changes in the transcript levels of neither *ALN* nor *AAH* in the nodules of any of the genotypes after 7 days of water stress. In contrast, *ALN* mRNA expression was induced in stems and leaves of the breeding lines PMB-0285 and PMB-0306 and in the PHA-0246 landrace, but not in PHA-0683, that showed similar expression levels in control and drought-treated tissues. Moreover, the expression of *ALN* mRNA was higher in the drought-sensitive than in tolerant genotypes (Fig. 4A). In contrast to *ALN*, the expression of *AAH* mRNA did not show any relevant change in tissues from drought-stressed plants,

compared to the well-irrigated controls, from any of the four genotypes (Fig. 4B).
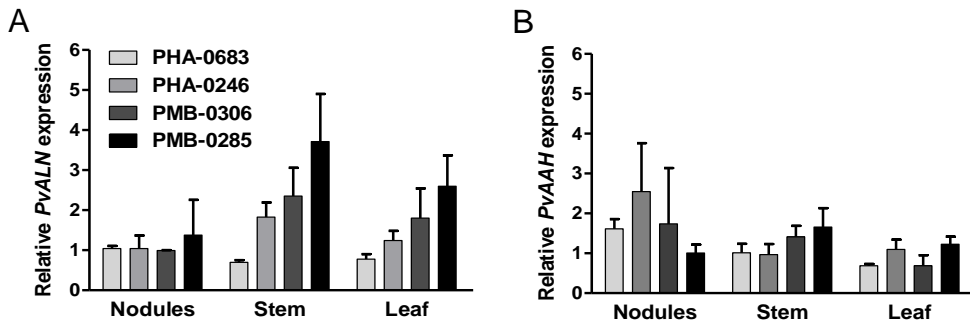
Fig. 4. Allantoinase (*ALN*) and allantoate amidohydrolase (*AAH*) gene expression in tissues from common bean genotypes after 7 days of water deficit. Relative transcript levels of (A) *ALN*, and (B) *PvAAH* genes in drought stressed tissues of PHA-0683, PHA- 0246, PMB-0306 and PMB-0285 common bean genotypes. Shown is the relative expression in samples from 7 days drought-stressed plants versus control, well irrigated ones. Data were normalized to the expression in the same tissues of *Actin-2* gene. Results are representative of at least three independent experiments, with three technical replicates for experiment.

4. Accumulation of ureides is mainly due to an induction of allantoinase activity

Besides gene expression levels, enzymatic activity of ALN and AAH, which catalyze the synthesis and degradation of allantoate, respectively, were measured in tissue samples from control and drought-stressed plants from all four accessions. From these two enzymes, ALN was determined in the same tissues in which ureides were determined, whereas AAH levels was only determined in leaves, because its activity level was too low for reliable measurement in other samples. ALN activity increased alongside with the progressive water deficit in aerial tissues (stems and leaves), whereas it was maintained at levels comparable to control samples in the roots and nodules from the stressed plants (Fig. 5). Moreover, ALN activity levels in shoots from the four genotypes followed a similar trend than the increments in ureide levels in the corresponding samples (Fig. 5C and 3C). In the stems of the stressed plants ALN induction level was similar among all four genotypes, and augmented with increasing stress (Fig. 5C). In contrast to the similar induction in stems in the four genotypes, leaves showed

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a differential pattern of ALN induction.
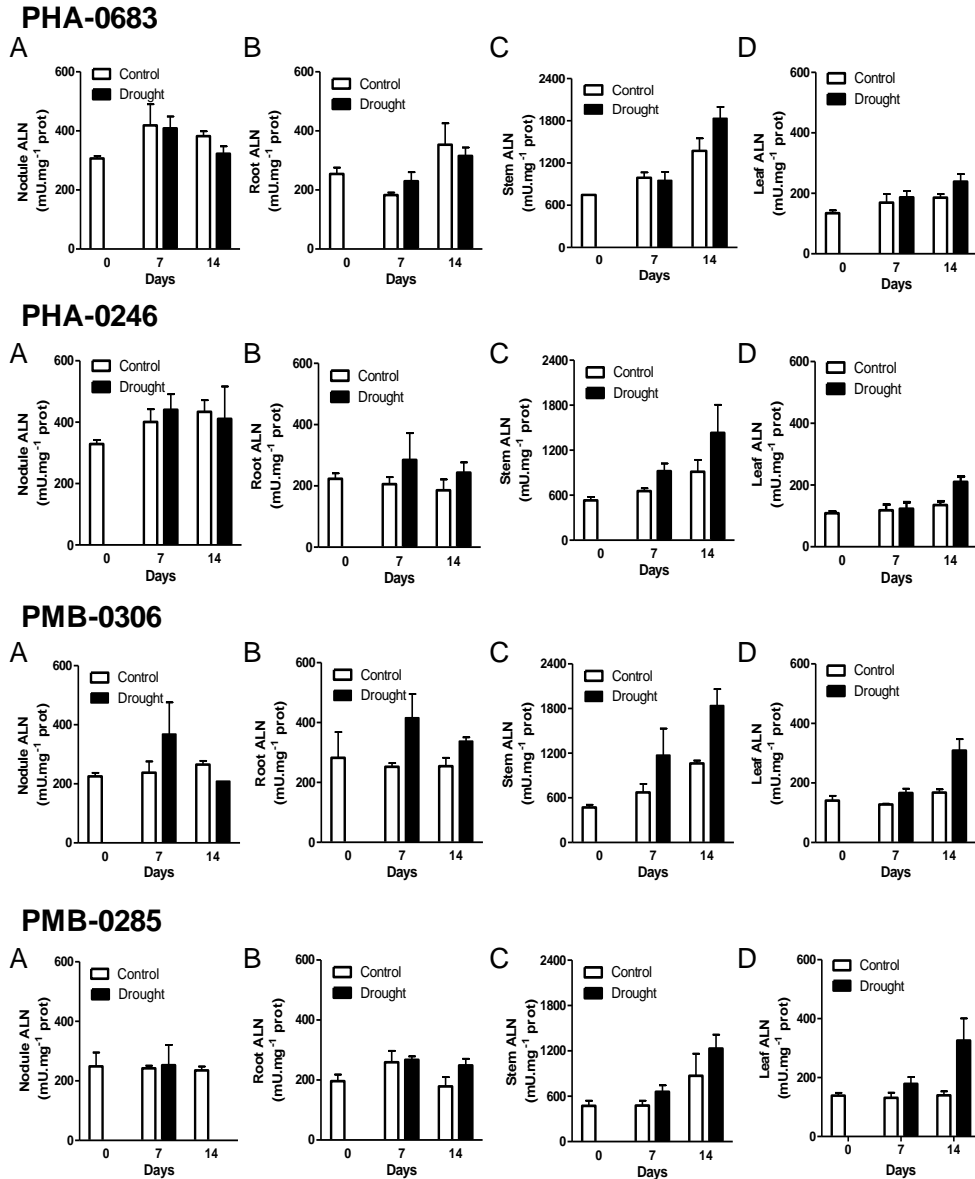
Fig. 5. Allantoinase (ALN) activity in tissues from common bean genotypes after 7 and 14 days of water deficit. Enzymatic assays were performed using crude extracts obtained from (A) nodules, (B) roots, (C) stems and (D) leaves of well watered (control) and drought-stressed plants (drought) at 0, 7 and 14 days after drought treatment. The enzymatic activities are represented as mU per mg of total soluble protein. Results are the mean of at least three independent experiments and each assay was done by duplicate (technical replicate).

After 7 days of drought-treatment, ALN activity in the stressed leaves of PHA-

0246 and PHA-0683 was similar to that in controls, whereas the leaves from PMB-0285 and PMB-0306 showed a slight ALN increment. Allantoinase activity was induced in all plant genotypes after 14 days of drought-treatment (Fig. 5D). Nevertheless, ALN activity levels at 14 days of stress showed a higher induction in the PMB-0285 and PMB-0306 than in the PHA-0246 and PHA-0683 (Fig. 5D). Remarkably, PMB-0285 and PMB-0306 were the lines responding to stress with the largest increments in leaf ureides and whose N_2 fixation was more sensitive to drought (Fig. 1 and 3D).

In contrast to the ALN induction in the stressed leaves, AAH activity was only slightly reduced under severe stress (14 days of water deprivation) in the leaves of the most drought-sensitive, PMB-0285 (Fig. 6).
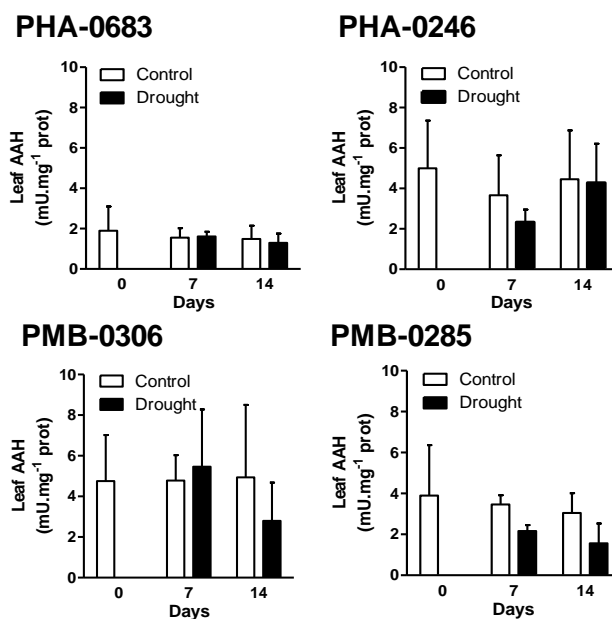
Fig. 6. **Allantoate amidohydrolase (AAH) activity in leaves from common bean genotypes after 7 and 14 days of water deficit.** Activity assays were done using crude extracts from leaves of well watered (control) and drought-stressed plants (drought) at 0, 7 and 14 days after drought treatment. The enzymatic activity is represented as specific activity (mU per mg of total soluble protein). Results are the mean of at least three independent experiments and assay was done by duplicate (technical replicate).

Discussion

Nitrogen fixation is among the more severely affected processes in situations of

water scarcity in legumes (Sinclair and Serraj, 1995; Zahran, 1999). Genotypic differences in N_2 fixation tolerance to drought have been reported in soybean (Sall and Sinclair, 1991; Purcell *et al.,* 1997; Serraj and Sinclair, 1996; 1997; Serraj *et al*., 1997) and also in common bean (Ramos *et al*., 1999; Teran and Singh, 2002; Riveiro, 2012; Devi *et al.*, 2013). The objective of this work has been to analyze whether the genotypic differences concerned not only the tolerance or sensitivity of the N_2 fixation process, but also the accumulation of ureides under these stress conditions.

Genotypic variations in the N_2 fixation response to water deficit have been found to be related to the accumulation of ureides in soybean genotypes (Sall and Sinclair, 1991; Serraj and Sinclair, 1996; Purcell *et al.*, 2000; Purcell *et al.*, 2004). However, in one of the few studies in which the N_2 fixation response and changes in ureide levels were compared among common bean genotypes there were no consistent variability in the N_2 fixation response to drought, although the varieties showed differences in the levels of ureides under such conditions (Serraj and Sinclair, 1998). The results presented here show that variability in N_2 fixation response to drought also takes place among common bean genotypes (Fig. 1). Comparison of the N_2 fixation sensitivity to drought in the nodulated roots from four common bean genotypes showed that landraces PHA-0246 and PHA-0683 were able to maintain higher N_2 fixation rates with moderate water stress than the breeding lines PMB-0285 and PMB-0306 (Fig. 1). From these, PMB-0306, considered as an improved tolerant breeding line, showed a moderate sensitivity, whereas, as expected, N_2 fixation was highly inhibited in the line PMB-0285, reported as sensitive (Frahm *et al.,* 2003) (Table 1).

In contrast to the differential effect of water stress on N_2 fixation, there were no substantial differences in the leaf relative water content (RWC) among the four genotypes (Fig. 2B). Moreover, comparison of nodule and plant biomass among control and stressed tissues showed that the drought treatment affected more severely to the nodules than to plant tissues (Table 3). Drought had little effect on plant growth in any of the genotypes, with a shoot biomass reduction after 14

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days of water deprivation ranging from 10-24% among sensitive and tolerant genotypes. However, despite the relatively mild effect on shoots, the water deficit treatment caused a severe dry weight reduction in the nodules of the most sensitive genotypes while the effect was moderate in the most tolerant ones (Table 3). This effect was fully coincident with the degree of N_2 fixation inhibition in the different accessions (Fig. 1). According to these results, in a similar study using a common bean genotype considered as drought tolerant, Ramos *et al*. (1999) reported a relatively small effect on both leaf water potential and plant dry mass after 10 days of exposure to two regimes of water deficit. These authors also found that nitrogenase activity and nodule dry mass were more affected by the treatment than the plant growth parameters.

Reports in soybean have shown that under drought stress a high increment of ureide concentrations is produced in the shoots of the most sensitive genotypes (Purcell *et al.*, 2000; Sinclair *et al.*, 2000), and it was proposed that ureides could be involved in the inhibition of N_2 fixation, by means of feedback signalling (; Serraj *et al.*, 1999; King and Purcell, 2005). In this work we observed that, in contrast to soybean, in the common bean genotypes analyzed, there was not a straightforward relationship between the accumulation of shoot ureides and drought sensitivity, since all four genotypes actually showed accumulation of ureides in their stems under drought conditions. Moreover, ureide concentrations increased in stems under water deficit independently of the degree of N_2 fixation tolerance or sensitivity to drought in each particular genotype (Fig. 1 and 3). Furthermore, N_2 fixation in the more tolerant genotypes was not inhibited by the ureides already accumulated in their stems at 7 days of treatment (Fig. 1 and 3). These data suggest that, contrary to reports in soybean, shoots ureide accumulation in common bean could not be the primary signal of N_2 fixation inhibition.

In contrast to the results in stems, we have found that ureide accumulation in stressed leaves is fully consistent with previous reports in soybean. There was a higher accumulation of ureides in the leaves from the genotypes in which N_2

fixation was more heavily inhibited than in the leaves of those in which N_2 fixation was more tolerant (Fig. 1 and 3).

There is still some controversy on whether the accumulation of ureides, and hence their supposedly inhibitory effect on N_2 fixation, occurs at a local level, in the nodules, or whether the increase of ureides in shoots may be able to inhibit nodule activity through systemic signalling. Several studies in soybean have suggested that the ureides accumulated in shoots would be transported by the phloem to nodules, where they inhibited nitrogenase activity (Vadez and Sinclair, 2001; Charlson *et al.*, 2009). Meanwhile, Ladrera *et al.* (2007) and King and Purcell (2005) attributed the N_2 fixation inhibition of sensitive soybean genotypes to the ureide accumulation at the local level, within nodules. Instead, in a recent work using a split-root system, the role of ureides as compounds causing N_2 fixation inhibition in soybean nodules was discarded, since, although ureide levels increased in the stressed nodules, the increment occurred later than the inhibition of N_2 fixation (Gil-Quintana *et al.*, 2013). In contrast to these studies, our results show that, in common bean, the ureide levels rise in aerial tissues (stem and leaves) but not in nodules along the experimental drought period (Fig. 3). These results are consistent with a recent work from our group, performed in the common bean breeding line 'Matterhorn' (Great Northern market class), in which accumulation of ureides in leaves and stems occurred even after the inhibition of N_2 fixation by the drought treatment, or even in the total absence of nodules, in nitrate-fed plants (Alamillo *et al.*, 2010). Accordingly, in this study, the near or total inhibition of N_2 fixation, with already senescent nodules, in the most sensitive genotypes (Fig. 1 and Table 3) was accompanied by progressive increases in ureide concentration in the stems and leaves (Fig. 3). Since the accumulation of ureides in the leaves occurred later than the N_2 fixation inhibition in the sensitive genotypes, this indicates that there is not an increment in the synthesis of ureides in nodules under such conditions (Fig. 1 and 3). Moreover ureides did not accumulate in nodules from either tolerant or sensitive genotypes. Lack of ureide accumulation in nodules subjected to drought stress

has been also observed in a recent study using a broad-range analysis of drought-induced metabolic adjustment in two common bean genotypes (Silvente *et al.*, 2012).

It remains an intriguing question why there is ureide accumulation in response to drought in the nodules of soybean susceptible genotypes but not in common bean ones, since both plants are ureidic legumes, and it would be interesting to check whether the symbiotic counterpart may have a contribution to these differences. The different kinetics of N_2 fixation inhibition and ureide accumulation shown in this and in our previous report further support that, at least in common bean, ureide accumulation in shoots does not seem to be the primary signal for nitrogenase inhibition in drought stressed nodules. Moreover, increase in ureide levels after nitrogenase inhibition suggest drought induction of signalling pathways that lead to ureide accumulation, probably through the remobilization of other compounds from senescent tissues.

King and Purcell (2005) proposed that an inhibition of allantoate amidohydrolase (AAH) activity mediated by Mn deficiency could be the actual cause of allantoate accumulation under drought. However, Charlson *et al.* (2009) did not found any relevant changes in AAH expression between controls and drought-stressed plants. Moreover, in common bean, neither AAH expression nor AAH activity changes were observed in response to drought but, in contrast, there was a high induction of allantoinase (ALN), the enzyme that catalyzes the synthesis of allantoate (Alamillo *et al.*, 2010). In this work we have observed that the rise in ureide concentration in the water-stressed leaves of the more sensitive genotypes was correlated with a higher induction of the expression and activity of ALN in these plants (Fig. 5). Nevertheless, in this work, we also found a slight inhibition of AAH activity in the most sensitive line (Fig. 6), although its expression was largely unaffected (Fig. 4B), thus suggesting that inhibition of allantoate degradation may also contribute to the high allantoate accumulation in the leaves of highly sensitive genotypes.

Treatments with ABA, the main hormone that regulates the plant response to

drought, induced the expression of key genes for the synthesis of ureides, as *XDH* (Yesbergenova *et al.*, 2005), *UO* and *ALN* genes (Alamillo *et al.*, 2010). Ureide synthesis induced in drought stressed leaves seems to be part of a general response mediated, at least in part, by ABA. This response probably follows the same pattern than N_2 fixation inhibition and, therefore, it could be related to drought sensitivity of each genotype. Results presented in this work suggest that there is not feasible that ureides accumulated in drought-stressed tissues might be involved in the feedback inhibition of N_2 fixation. Lack of ureides accumulation in the nodules and the increment in ureide levels after the inhibition of N_2 fixation in the more sensitive genotypes strongly suggests that ureides accumulating in leaves can not be solely originated in the nodules from stressed plants. In contrast, our results indicate that, under conditions of water deficit, alternative pathways of ureide production are activated in aerial tissues from common bean. In this sense, it is noteworthy that higher levels of allantoin have been observed in desiccation-tolerant plant *Sporobolus stafianus* compared to the desiccation-sensitive *Sporobolus pyramidalis* (Oliver *et al.*, 2011) and also in the dry stages of the extremely drought-tolerant moss *Sellaginella lepidophylla* (Yobi *et al.*, 2013). Similarly, high levels of grain allantoin in some rice genotypes have been shown as beneficial to withstand drought and other abiotic stresses (Wang *et al.*, 2012), pointing to nitrogen-rich ureides as part of a ancient and broad-range plant strategies to survive drought stress. Ureides have been suggested as the best compounds to channel remobilized nitrogen to seed filling (Díaz-Leal *et al.*, 2012). Therefore, it is tempting to speculate that they may act as nitrogen and perhaps carbon source to accelerate seed production under drought conditions. Nevertheless, further studies will be needed to ascertain the actual function of the accumulation of ureides under stress conditions.

In summary, the results shown here indicate that, like in soybean, there are large genotypic differences in the drought sensitivity of N_2 fixation among common bean germplasm, and that the genetic variation is linked to the ureide accumulation in stressed leaves. In addition, we have found that the two landraces

studied (PHA-0246 and PHA-0683) had a better performance under drought stress that the tolerant breeding line (PMB-0306), therefore they could be used in breeding programs aimed to improve the drought tolerance and the efficiency of the BNF in common bean.

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Supplementary material

Supplementary Fig. S1. Seeds of the four genotypes used in this study.

Supplementary Fig. S2. Effect of water stress in the appearance of the nodules along the treatment.

Supplementary Table S1. Student´s t analysis of apparent nitrogenase activity (ANA) and leaf ureide concentration (LUC) between control and drought-stressed plants of common bean genotypes after 7 and 14 days of water deficit.

Supplementary Table S2. Analysis of variance of apparent nitrogenase activity (ANA) and leaf ureide concentration (LUC) among common bean genotypes after 7 and 14 days of water deprivation.

Supplementary Table S3. LSD analysis of apparent nitrogenase activity (ANA) and leaf ureide concentration (LUC) of well-watered (control) and drought-stressed plants at 7 and 14 days of treatment in PHA-0683, PHA-0246, PMB-0306 and PMB-0285 common bean genotypes.

PHA-0683 PHA-0246 PMB-0306 PMB-0285

Supplementary Fig. S1. Seeds of the four genotypes used in this study.

PHA-0683

Drought days

| 0 | 7 | 14 |

PHA-0246

Drought days

| 0 | 7 | 14 |

PHA-0306

Drought days

| 0 | 7 | 14 |

PHA-0285

Drought days

| 0 | 7 | 14 |
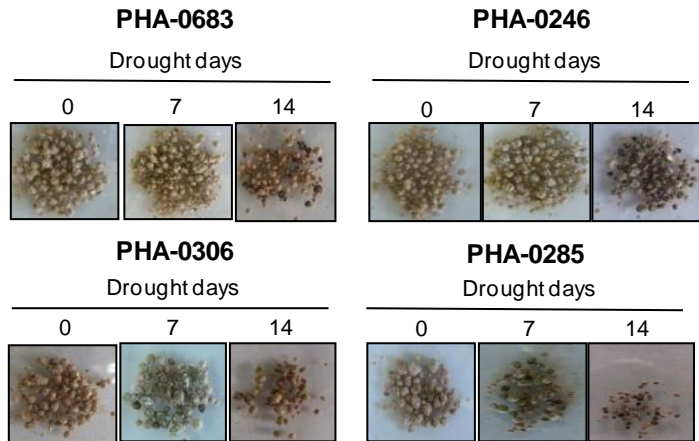
Supplementary Fig. S2. Effect of water stress in the appearance of the nodules along the treatment.

Supplementary Table S1. Student´s t analysis of apparent nitrogenase activity (ANA) and leaf ureide concentration (LUC) between control and drought-stressed plants at 7 and 14 days among common bean genotypes ($p < 0.05$ denotes significant differences, $p < 0.001$ highly significant differences).

Genotype	Sample	Apparent nitrogenase activity (ANA) (μmol H$_2$.h^{-1} plant)				Leaf ureide concentration (LUC) (μmol.g^{-1} DW)			
		7 days		14 days		7 days		14 days	
		Mean±SEM	p	Mean±SEM	P	Mean±SEM	p	Mean±SEM	p
PHA-0683	Control	2.780±0.36	0.1842	1.860±0.22	0.0110	7.472±1.79	0.4881	10.698±2.56	0.8877
	Drought	2.467±0.32		1.034±0.17		10.423±3.64		10.204±2.16	
PHA-0246	Control	2.217±0.27	0.4572	1.653±0.22	0.0109	4.900±0.72	0.0579	8.918±4.37	0.3936
	Drought	2.213±0.24		0.884±0.12		9.206±1.69		26.296±18.4	
PMB-0306	Control	2.579±0.29	0.1603	1.648±0.21	0.0002	14.402±3.03	0.1618	17.435±3.18	0.0024
	Drought	1.992±0.26		0.333±0.16		26.262±7.24		82.658±12.6	
PMB-0285	Control	2.953±0.34	0.0003	2.236±0.30	0.0001	10.907±3.45	0.2997	2.954 ±0.48	0.0001
	Drought	1.353±0.21		0.100±0.00		18.516±5.36		88.200±10.8	

*SEM: Standard Error Mean

Supplementary Table S2. Analysis of variance of (A) apparent nitrogenase activity (ANA) and (B) leaf ureide concentration (LUC) among common bean genotypes after 7 and 14 days of water deprivation. Significant differences are denoted by asterisks (* $p<0.05$, ** $p<0.01$).

(A) Apparent nitrogenase activity (ANA)																		
SOURCES OF VARIATION	Control 7 days			Drought 7days			C 7 d - D 7 d			Control 14 days			Drought 14 days			C 14 d - D 14 d		
	DF	MS		DF	MS		DF	MS		DF	MS		DF	MS		DF	MS	
Genotypes	3	0.48	ns	3	1.26	*	3	2.33	ns	3	0.99	ns	3	2.06	**	3	5.00	**
Replications	1	1.89	ns	1	1.64	ns	1	0.01	ns	1	0.15	ns	1	0.12	ns	1	0.00	ns
Individuals	11	1.69	ns	11	1.31	*	11	0.47	ns	10	0.90	ns	10	0.11	ns	10	1.01	*
Error	28	1.27		28	0.51		28	1.07		22	0.43		22	0.17		22	0.40	

(B) Leaf ureide concentration (LUC)																		
SOURCES OF VARIATION	Control 7 days			Drought 7 days			C 7 d - D 7 d			Control 14 days			Drought 14 days			C 14 d - D 14 d		
	DF	MS		DF	MS		DF	MS		DF	MS		DF	MS		DF	MS	
Genotypes	3	0.011	*	3	0.050	*	3	0.018	ns	3	0.020	*	3	0.462	*	3	0.388	*
Replications	1	0.001	ns	1	0.002	ns	1	0.000	ns	1	0.004	ns	1	0.005	ns	1	0.000	ns
Individuals	4	0.002	ns	4	0.001	ns	4	0.002	ns	3	0.006	ns	3	0.029	ns	3	0.016	ns
Error	7	0.002		7	0.008		7	0.010		6	0.004		6	0.071		6	0.070	

Supplementary Table S3. LSD analysis of (A) apparent nitrogenase activity (ANA) and (B) leaf ureide concentration (LUC) of well-watered (control) and drought-stressed plants at 7 and 14 days of treatment in PHA-0683, PHA-0246, PMB-0306 and PMB-0285 common bean genotypes.

(A) Apparent nitrogenase activity (ANA) (μmol H$_2$.h^{-1} plant)				
Genotypes	**Mean control 7 days**	**Mean drought 7 days**	**Mean drought 14 days**	**Mean C 14 d - D 14 d**
PHA-0246	2.500 a	2.217 ab	0.885 a	0.668 c
PHA-0683	2.830 a	2.471 a	1.003 a	0.858 bc
PMB-0285	2.950 a	1.687 b	0.010 b	2.227 a
PMB-0306	2.580 a	1.992 ab	0.229 b	1.433 b

(B) Leaf ureide concentration (LUC) (μmol.mg^{-1} prot)					
Genotypes	**Mean control 7 days**	**Mean drought 7 days**	**Mean control 14 days**	**Mean drought 14 days**	**Mean C 14 d - D14 d**
PHA-0246	0.071 b	0.125 b	0.092 b	0.270 b	-0.178 a
PHA-0683	0.084 b	0.116 b	0.139 ab	0.251 b	-0.112 a
PMB-0285	0.124 ab	0.349 a	0.065 b	0.843 a	-0.777 b
PMB-0306	0.182 a	0.353 a	0.250 a	0.983 a	-0.733 b

CHAPTER 3: ANALYSIS OF XANTHINE DEHYDROGENASE EXPRESSION AND ACTIVITY IN RESPONSE TO STRESS AND STRESS-RELATED MOLECULES

Abstract

Xanthine dehydrogenase is essential in the assimilation of the nitrogen fixed by the ureidic legumes. Uric acid, produced in the reaction catalyzed by XDH, is the precursor of the ureides, allantoin and allantoate, which are the main N-transporting molecules in these plants. There is increasing number of studies involving XDH and ureides with stress responses. However, the physiological role of XDH under stressful conditions in ureidic legumes remains largely unexplored. *In silico* analysis of the upstream genomic region of XDH gene from *Phaseolus vulgaris* showed the presence of several stress-related *cis*-regulatory elements. The functionality of the regulatory motifs was checked by the analysis of the *PvXDH* mRNA in plants treated with stress-related phytohormones and subjected to drought, dehydration and extreme temperatures. *In vitro* assays showed that PvXDH protein can act as a XDH, XO and NADH oxidase, although the XDH was the major activity, and the only regulated by stress, suggesting that probably XDH is the only relevant activity of this protein *in vivo*. PvXDH activity is inhibited by NO, probably through a mechanism mediated by peroxynitrite and iron, in tissues others than nodules, where uric acid may serve as protective molecule of XDH activity in *P. vulgaris*.

Introduction

Xanthine oxidoreductase (XOR; EC 1.17.1.4, formerly 1.1.1.204) is a FAD-, molybdenum-, iron- and sulfur-containing hydroxylase with a pivotal role in nucleotide metabolism in all organisms. The enzyme catalyzes the oxidation of hypoxanthine to xanthine and the oxidation of xanthine to uric acid (Harrison, 2002). It is a dimer of about 300 kDa, with two identical subunits, each composed of three characteristic domains: two non identical iron-sulfur clusters of the [2Fe-2S] type at the N-terminus, followed by a FAD-binding domain and a long C-terminal domain comprising a dimerization domain plus a molybdenum cofactor (Moco) binding site (Hille and Nishino, 1995). In mammals, XOR enzyme exists in two forms, xanthine dehydrogenase (XDH; EC 1.17.1.4) and xanthine oxidase (XO; EC 1.1.3.22). Both enzymes are encoded by the same gene but XDH is converted to XO by the reversible oxidation of two cysteine residues (Corte and Stirpe, 1972; Nishino and Nishino, 1997), or by irreversible proteolysis of a C-terminal peptide (Amaya *et al.* 1990).

In animal tissues, the predominant form under physiological conditions is XDH, which preferentially uses NAD^+ as electron acceptor, although it can also reduce O_2 with lower affinity when NADH is used as the electron donor (Sanders *et al*., 2006). Instead, XO form transfers the reducing equivalents directly to O_2, generating superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2). Through its O_2^--generating activity, XOR has been considered a relevant enzyme for cell signaling and in tissue damage processes (Harrison, 2002). The biochemical properties (molecular mass, cofactors and substrate preference) of XDHs from higher plants are similar to the corresponding enzymes from mammals, as it was showed in XDH proteins from wheat (Montalbini, 1998) and some legumes (Boland, 1981; Triplett *et al*., 1982; Montalbini, 2000; Sauer *et al.,* 2002). However, in contrast to animals, XORs from plant sources have been found only in the XDH form and lack the key cysteine residues (Cys-535 and Cys-992) involved in the reversible conversion of XDH into XO (Hesberg *et al*., 2004).

XDH is a key enzyme in purine nucleotide catabolism and ureide synthesis, since it catalyzes the first and rate-limiting reaction of the degradation of purines, which leads to production of the ureides (Stasolla *et al.,* 2003; Zrenner *et al.,* 2006). Early work on XDH in plants were done in legumes, although they were limited to the purification and partial biochemical characterization of the XDH protein from bean nodules (Boland, 1981; Triplett *et al.,* 1982), leaves of different legume species (Montalbini, 2000) and pea seedlings (Sauer *et al.,* 2002). In contrast, studies concerning the molecular analysis of XDH from legumes have not been presented so far, and information is limited to the sequence data available in public resources (NCBI; Phytozome). Molecular characterization of genes coding XDH in plants has only been done in *Arabidopsis thaliana*. There are two copies of XDH gene in Arabidopsis; *AtXDH1*, whose expression is regulated by developmental and stress conditions, and *AtXDH2*, with a low and constitutive expression level (Hesberg *et al.,* 2004). Expression of *AtXDH1* was found to be induced during natural senescence, according to the high purine degradation and remobilization of nitrogen sources under these conditions (Zrenner *et al.,* 2006). Induction of XDH under natural senescence was also observed in pea (Pastori and Del Rio, 1997). Moreover, Nakagawa *et al.* (2007) showed that *AtXDH1* silencing caused developmental defects as retarded growth, defects in fertility and early senescence in the antisense transgenic plants, which were rescued by uric acid supplementation, suggesting a requirement of purine catabolites for sustaining normal plant growth and development.

Apart from the important function regarding the recycling of carbon and nitrogen in the cell, other roles related with the response to biotic and abiotic stresses have been assigned to plant XDHs. Among others, XDH has been implicated in plant-pathogen interactions (Montalbini, 1992a, b) and in the hypersensitive response associated with this (Montalbini, 1995). In *A. thaliana*, stressful conditions such as drought, low temperatures, salinity and extended darkness, lead to the induction of *AtXDH1* gene expression and protein activity (Hesberg *et al.,* 2004; Yesbergenova *et al.,* 2005; Brychkova *et al.,* 2008; Watanabe *et al.,* 2010;), and

this induction was shown to be mediated by the stress-related hormone ABA (Hesberg *et al.,* 2004).

Moreover, some studies have also shown that XDHs from plants are able to produce O_2^- by using xanthine/hypoxanthine or NADH as electron donors and O_2 as acceptor (Hesberg *et al.,* 2004; Yesbergenova *et al.,* 2005; Brychkova *et al*., 2008; Zarepour *et al.,* 2010), suggesting that this enzyme might contribute to the production of ROS during plant ageing and stress conditions.

In this work, the full-length cDNA and the regulatory upstream sequences of XDH gene from *P. vulgaris* has been isolated. Gene expression and enzyme activity levels in response to drought stress and several stress-related conditions have been analyzed. Moreover, the possible contribution of the enzyme to ROS production was studied. We show that PvXDH activity is subjected to *in vitro* inhibition by nitric oxide (NO) and the role of uric acid protecting XDH from the inhibition is discussed.

Materials and methods

1. Plant material, growth conditions and *in vivo* treatments

Seeds of *Phaseolus vulgaris* L. genotypes PHA-0683, Great Northern and PHB-0285 were surface-sterilized by sequential dipping in ethanol (30 s) and 0.2% (w/v) sodium hypochlorite (5 min) and then washed thoroughly with distilled water. Soaked seeds were allowed to germinate in Petri dishes (120 mm diameter) with wet paper under sterile conditions. After germination, 4 seedlings were sown on each pot (16 cm diameter, 18 cm height) filled with an artificial substrate composed of vermiculite/perlite mixture (2/1 w/w) and inoculated with a fresh suspension of *Rhizobium leguminosarum* ISP 14**,** which was cultured at low temperatures (28 °C) for less than 30 hours. Inoculated plants were watered three times a week with a nitrogen-free nutrient solution (Rigaud and Puppo, 1975). Plants were cultured in growth chamber under long-day photoperiod (16 hours light, 8 hours dark), 300 $\mu E.m^{-2}.s^{-1}$ lighting, and 70% relative humidity at 26-21

°C (day-night temperatures) (Alamillo *et al.,* 2010).

In vivo PvXDH analyses were performed in plants exposed to the following treatments. (i) *Drought stress:* water stress treatments were performed as in (Chapter 2) using the PHA-0683 (Tolerant), Great Northern (Intermediate) and PMB-0285 (Sensitive) common bean varieties (Riveiro, 2012). Briefly, at the onset of flowering, which happened at 25–28 days after sowing (according to bolting dates of each genotype), plants were randomly separated into two sets. Watering was avoided to one half of the plants and the second half was regularly irrigated. Plant collection was done at 7 days from the beginning of the treatment in both, control and water-stressed plants. (ii) *Treatment with phytohormones:* The leaves from plants at 26 days after sowing of the Great Northern variety were treated with 10 µM abscisic acid (ABA), 50 µM jasmonic acid (JA), 200 µM salicylic acid (SA), 2 µM 1-aminocyclopropane-1-carboxylic acid (ACC), 2 µM gibberellic acid (GA) or 200 µM sodium nitroprusside (SNP). Stock solutions were prepared in 2 mL of ethanol (ABA, JA, GA) or 2 mL of distilled water (SA, ACC, SNP), and diluted with the nitrogen-free nutrient solution to achieve the final working concentrations. Leaves were sprayed twice at 48 and 24 hours before harvesting. Control plants were treated with distilled water or with ethanol at concentration similar to the one used to prepare working solutions of phytohormones. All compounds were purchased from Sigma-Aldrich. (iii) *High temperature (39 ºC), low temperature (4 ºC) and dehydration*: Leaves from plants at 28 days after sowing of the Great Northern variety were detached and incubated at 39 ºC for 2 hours, at 4 ºC for 4 hours or dehydrated until a 50% loss in fresh weight. Detached leaves used as a control were incubated in Petri dishes (120 mm diameter) on moist paper to prevent dehydration, during the same time as their respective treatments. (iv) *In planta SNP treatment:* Plants at 26 days after sowing of the Great Northern variety were irrigated with the nitrogen-free nutrient solution containing 0.5 or 1 mM SNP at 48 and 24 hours before harvesting.

Plant material was collected at indicated times after the treatments, frozen with

liquid nitrogen and stored at -80 ℃ until further analysis.

2. *In vitro* treatments

For *in vitro* treatments, crude extracts were incubated with the corresponding concentration of the indicated chemical for 30 min at 30 ℃ in darkness. Depending of the experiment, the following concentrations of chemicals were used: 0.1, 0.5 or 1 mM of the NO-donors sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO) and S-nitroso-N-acetyl-DL-penicillamine (SNAP); (0.01, 0.1 or 1 mM $FeSO_4$; 0.1 mM potassium ferricyanide (FeCy); 0.1 mM hypoxanthine and 0.5 μM uric acid.

Where indicated, after the incubations with SNP or FeCy, 10 mM of the reducing agent dithiothreitol (DTT) was added and incubated on ice for 30 min, as control of the irreversible modification of the protein.

NO-donors stock solutions (5 mM) were prepared dissolving GSNO and SNP in water and SNAP in DMSO. FeCy stock solution (5 mM) was prepared in distilled water. $FeSO_4$ stock solution (10 mM) was prepared in distilled water. Hypoxanthine and uric acid stock solutions (1 mM) were prepared in 50 mM Tris-HCl, pH 8. All chemicals were purchased from Sigma-Aldrich. Stock and working solutions were prepared immediately before use.

3. Determination of enzymatic activities

All procedures for crude extract preparation and enzyme manipulations were carried out at 0–4 °C. Frozen plant material was ground to a fine powder under liquid nitrogen. Plant extracts were obtained by adding 3 mL of extraction buffer (2.5 mM EDTA, 5 mM DTT, 20 μM FAD in 100 mM potassium phosphate at pH 7.5) per gram of tissue. The resulting homogenate was centrifuged at 15000 *g* for 10 min and the supernatant was used for activity assays

3.1 In-gel determination of enzymatic activities

As a routine 5, 10 and 50 μg total protein from nodules, roots and leaves,

respectively were loaded. Proteins were separated at 4 °C in 7% (w/v) native polyacrylamide gels under non-reducing conditions. After native electrophoresis, xanthine dehydrogenase (XDH) activity was detected by incubation of the gels in a reaction mixture containing 1 mM hypoxanthine, 1 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 0.1 mM phenazine methosulfate (PMS) in 0.1 M Tris-HCl buffer, pH 8.5 at 25 °C in the dark. Xanthine oxidase (XO) and NADH oxidase (NADHox) activities were detected in gels for their ability to generate O_2^- by the reduction of nitroblue tetrazolium (NBT) as described in Sagi and Fluhr (2001). Briefly, the gels were incubated in a reaction mixture containing 1 mM hypoxanthine for XO activity or 0.25 mM for NADHox activity, and 0.3 mM nitro-blue tetrazolium chloride (NBT) as electron acceptor; in 0.1 M Tris-HCl buffer, pH 7.5 at 25 °C in the dark. Where indicated, superoxide dismutase (SOD) (60 mU.mL^{-1}) or allopurinol (500 μM) were added to the reaction mixture. The reactions were stopped by dipping of the gels in 7% acetic acid. The quantity of the resulting formazan bands formed by the reduction of tretrazolium salts (MTT and NBT) was directly proportional to enzyme activity during a given incubation time (Rothe, 1974). In-gel determinations of XDH activity in crude extracts incubated with NO-donors were carried out both in non-dialyzed and in dialyzed extracts. The dialysis was done using *SpinTrap G25* columns previously equilibrated with the extraction buffer.

Quantitative analyses of band staining were made using Quantity One software (Bio-Rad). Representative gel images from one of at least 3 independent experiments are shown in the figures.

3.2. Spectrophotometric determination of XDH activity

XDH activity was measured in dialyzed extracts at 30 °C in 1 mL reaction mixture containing 0.6 mM xanthine, 1 mM NAD$^+$, 20 μM FAD in 50 mM Tris-HCl at pH 8.0; and the appropriate amount of plant extract. Activity of each sample was measured during 5 min following the linear production of NADH at 340 nm.

Soluble protein was measured in the same extracts in which the enzymatic activities were determined (Bradford, 1976). The results are expressed as means of the values from at least three independent measurements.

4. Isolation of full-length PvXDH coding sequence

Gene specific primers (Supplementary Table S1) were designed according to ESTs sequences from *P. vulgaris* with high similarity to the XDH sequences from *Glycine max* (XM_006594977 and XM_006598231) available in the database (GenBank) at the beginning of this investigation. Total RNA and cDNA were prepared from nodules of the Great Northern genotype, and used as a template for *PvXDH* amplification. The resulting PCR fragment was cloned into pSparkI vector (Canvax Biotech SL) and the sequence from several independent clones was determined. Later on, the identity of the full cDNA sequence obtained was confirmed by the recently released whole genome sequences (http://phytozome.jgi.doe.gov/) (Schmutz *et al.,* 2014).

5. *PvXDH* promoter sequence isolation

The proximal promoter sequence of *PvXDH* gene was isolated using the *GENOME WALKER^{TM} Universal kit* (Clontech) and the transcription start site was determined by the isolation of the 5´-UTR sequence with the *SMART^{TM}RACE* kit (*Rapid Amplification of cDNA-ends*, Clontech) following the manufacture´s instructions. Gene inner specific primers nearly to 5´ end (Supplementary Table S1) were designed according the *PvXDH* cDNA sequence cloned previously. Two consecutive PCRs were done, according to manufacturer instructions. The resulting DNA fragments were cloned into pGEMt-easy vector (Promega, Madison WI) or pSparkI vector (Canvax Biotech SL) and the sequence from several independent clones was determined.

6. Gene expression analysis

Total RNA was isolated from the different tissues using the TRI REAGENT (Sigma-Aldrich) following the manufacturer's instructions. Prior to RT-PCR, total RNA from control and treated tissues was treated with RNAase-free DNAaseI (Promega, Madison WI) at 37 ºC for 30 min to eliminate any traces of genomic DNA. Lack of 18S rRNA amplification was used to check the successful removal of DNA. First strand cDNA synthesis was done with 2.5 µg of DNAase-treated RNA using iScript™ reverse transcriptase (Bio-Rad) following the manufacturer's instructions. Expression analysis was performed by quantitative RT-PCR in an iCycleriQ System (Bio-Rad, Hercules, CA, USA) using the iQ SYBR-Green Supermix (Bio-Rad) and *PvXDH* specific primers (Supplementary Table S1). The PCR programme consisted of an initial denaturation and Taq polymerase activation step of 5 min at 95 ºC, followed by 50 cycles of 15 s at 95 ºC and 1 min at 60 ºC. Expression of *Actin-2* and 18S genes was used as internal controls, after checking that its level of expression was similar in all samples. Specificity of primers was confirmed by dissociation curves analysis and by sequencing of the PCR products. The amplification efficiency of each primer pairs, calculated by PCR using serial dilutions of root and leaf cDNAs, was > 98%. Analysis of relative gene expression was calculated from ΔCT values (Livak and Schmittgen, 2001), using expression of *Actin-2* gene for data normalization. All the reactions were set up in triplicate (three technical replicates) using at least three RNA preparations from tissues collected from independent experiments (biological replicates).

7. Western blot analysis

Antibodies against PvXDH were raised in rabbits immunized with two small PvXDH peptides produced in bacteria. The peptides span positions 136-239 (103 amino acids) and 1205-1342 (137 amino acids) at the PvXDH protein. The sequences of the two peptides were PCR amplified and cloned into the bacterial expression vector pET30b+ (Novagen), according to reported procedures (Díaz-

Leal *et al*., 2014), using the primers listed in Supplementary Table S1. A mixture of the purified recombinant peptides was used to raise the antibodies in rabbits using the facilities of the SCAI-UCO.

Proteins were separated by 7% SDS-PAGE (Laemmli, 1970) in a Mini PROTEAN III system (BioRad). After separation, the proteins were electro-transferred to polyvinylidene fluoride membrane (Sigma). To detect PvXDH protein, blots were incubated with anti-PvXDH polyclonal antibodies at a 1:1000 dilution. Anti-rabbit IG, alkaline phosphatase-conjugated (Sigma) was used as secondary antibody at a 1:12000 dilution and the reaction was developed using 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (BCIP) and NBT. The specificity of the antibodies was tested by immunolablelling with the preimmune serum.

8. Nitrogen fixation measurement

Nitrogenase (EC 1.7.9.92) activity was measured as the representative H_2 evolution in an open-flow system (Witty and Minchin, 1998) using an electrochemical H_2 sensor (Qubit System Inc., Canada). For that, nodulated roots excised from their shoots just before the activity assay were sealed in 0.125 L cylinders and H_2 production was recorded according to the manufacturer´s instructions. Apparent nitrogenase activity (ANA, rate of H_2 generation in air) was determined under $N_2:O_2$ (80%:20%) with a total flow of 0.4 L.min^{-1}. After reaching steady-state conditions, total nitrogenase activity (TNA) was determined under $Ar:O_2$ (79%:21%). Standards of high-purity H_2 were used to calibrate the detector. Roots attached to whole plants were also measured to ascertain that the separation from shoots did not cause a significant reduction on nodule activity during the assay. Data are given as N_2 fixed per plant.

9. Silencing of *PvXDH* expression and plant transformation

To generate the silencing hairpin RNA construct, a fragment from the 3´ region of *PvXDH* cDNA was cloned into pFGC5941 vector (AY310901). For that, 362 bp

of cDNA were amplified by PCR using a forward primer containing the *Xba*I and *Asc*I restriction sites and a reverse primer containing the *Bam*HI and *Swa*I restriction sites (Supplementary Table S1). The resulting PCR product was cloned using *Asc*I and *Swa*I sites to insert the fragment in sense orientation into pFGC5941, upstream of an intron, and the *Bam*HI and *Xba*I sites for cloning the fragment in antisense orientation, downstream of the intron. The resulting construct was verified by sequencing and introduced into *Agrobacterium rhizogenes* strain K599. Common bean plantlets were transformed according to (Estrada-Navarrete *et al.,* 2007). Briefly, Great Northern seeds were surface sterilized and germinated in Petri dishes with moist paper under dark conditions at 26-21ºC. Three days after germination, seeds were planted in pots filled with wet vermiculite and cultivated under standard growth conditions. Three days later, a dense suspension of *A. rhizogenes* carrying the silencing construct, or the empty pFGC5941 vector as a control, were injected into the cotyledonary nodes. After the inoculation, the plants were placed into a chamber with >90% relative humidity. Plants were regularly watered and sprayed with B&D solution (Estrada-Navarrete et al., 2007) supplemented with 0.5 mM KNO_3. After about two weeks, hairy roots generated in the wounds were about 2 cm long. Once hairy roots were long enough, the primary root of the plants was excised by cutting approximately 1 cm below the cotyledonary nodes, and plants were sown and inoculated with *Rhizobium tropici* strain CIAT899, by adding 1 mL of fresh culture directly to the transgenic roots. Plants were watered with B&D nitrogen-free solution and about four weeks later roots and nodules were collected and frozen in liquid nitrogen.

10. Statistical analysis of the data

ANOVA and *Student´s t*-test statistical analysis were performed using the GraphPad software package.

Results

1. Sequence of full-length *PvXDH* cDNA

A full-length cDNA of *PvXDH* with an open reading frame of 4089 bp was obtained by RT-PCR using available sequence information of the *PvXDH* ESTs in the NCBI database. The longest deduced ORF encoded a protein of 1362 amino acids, with a 49% similarity to the human XDH and a 76.4% and 74.7% similarity to the Arabidopsis proteins XDH1 and XDH2, respectively (Supplementary Fig. S1). Comparison of PvXDH primary structure with homologous proteins from other organisms revealed that XDH protein from *P. vulgaris* shared the typical three-domain structure found in XOR proteins (Fig. 1 and Supplementary Fig. S1). It contained an N-terminal domain spanning amino acids 24 to 175 that included eight strictly conserved cysteine residues required for binding of two non-identical iron-sulfur clusters of the [2Fe-2S] type. Following the two [2Fe-2S] cluster domain and separated by a less conserved region was the characteristic FAD-binding domain (amino acids 267 to 443), showing the highly conserved FFLPGYR motif (amino acids 420 to 426), supposed to be the binding site of the substrate NAD^+ via Tyr_{419} residue (Amaya *et al.,* 1990; Nishino and Nishino, 1987). The third recognized domain was the C-terminal molybdenum cofactor (Moco, molybdopterin) spanning amino acids 728 to 1273. This domain was separated from the FAD-binding domain by another hinge region and contained the dimerization motif and the hypoxanthine/xanthine-binding site. Conserved glutamate and arginine residues (Glu-832 and Arg-910), supposed to be essential for interaction with purine substrates (Glatigny *et al.,* 1998) were also present in the deduced sequence. In contrast to the large conservation of essential motifs, the protein from *P. vulgaris*, as well as its two analogues in Arabidopsis, lacked the essential cyteine residues and the C-terminal peptide, which should be modified or cleaved upon XDH conversion into the XO form in animals (Nishino and Nishino, 1997; Nishino *et al.,* 2008; Nishino and Okamoto, 2015) (Supplementary Fig. S1).
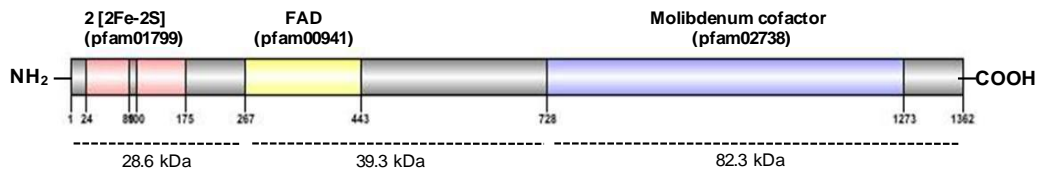
Fig. 1. Organization of peptide domains in the xanthine dehydrogenase (XDH) protein from *P. vulgaris*. Positions of the three characteristic functional domains: [2Fe-2S], FAD, and Moco binding domains (PFAM: 01799, 00941 and 02738, respectively) are indicated.

2. Expression and activity of XDH in tissues from *P. vulgaris*

Expression of *PvXDH* mRNA was analyzed in nodules, roots and leaves from plants of the Great Northern genotype collected at 30 days after sowing. As is shown in Fig. 2A, the transcript expression level in nodules was about 20-fold higher than in leaves, and about 8-fold higher than in roots. Accordingly, XDH activity in these tissues followed a similar pattern. Using in-gel assay and similar amounts of total protein per sample, the highest band intensity of XDH activity was found in nodules, corresponding to about 5-fold higher activity than in roots and about 20-fold higher than in leaves (Fig. 2B). Western-blot analysis corroborated the differences in the level of PvXDH (Fig. 2C), which indicated that XDH level is higher in nodules than in any other tissues and that it is transcriptionally regulated in common bean tissues. The high XDH activity in nodule extracts could also be confirmed by the spectrophotometric measurement, whereas activity in leaf extracts was below the detection level in this assay, which is explained by the approximately 20-fold lower sensitivity of the spectrophotometric determination, in comparison to the in-gel electrophoretic assay (Supplementary Fig. S2). Due to the large difference in the activity level among leaves and nodules, the linearity of the in-gel XDH assay was ascertained using increasing protein concentrations (Supplementary Fig. S2) and loading amounts of 5, 10 and 50 µg total protein from crude extracts from nodules, roots and leaves, respectively, were chosen as adequate protein levels to visualize the in-gel XDH activity in further experiments.
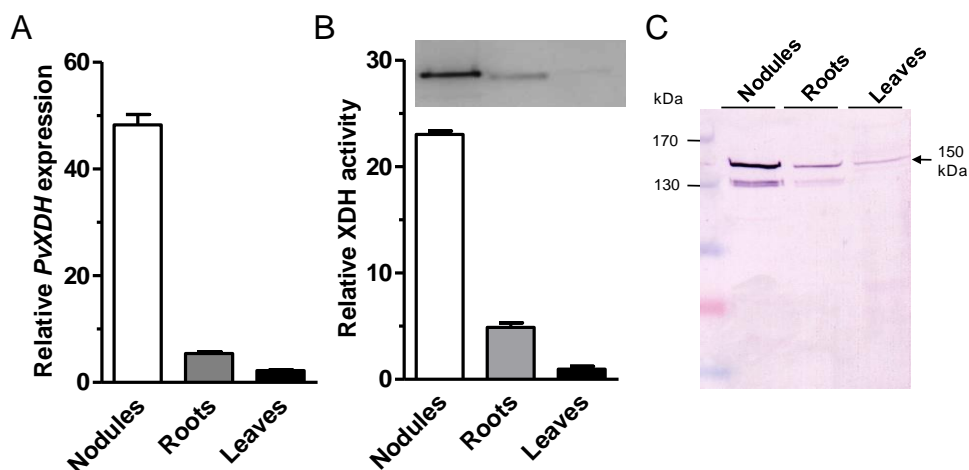
Fig. 2. XDH expression, activity and protein in tissues from *P. vulgaris*. (A) XDH mRNA expression (B) XDH activity (Arbitrary Units) and (C) XDH protein levels in nodules, roots and leaves from plants of 30 days after sowing cultured under N_2 fixation conditions. Expression data were normalized according to *Actin-2* gene expression. For in-gel activity assay, 7.5 µg of total soluble protein from crude extracts from nodules, roots and leaves were loaded. Quantitative analyses were done using Quantity One software (Bio-Rad). For western blot analysis, 7.5 µg protein from nodules and roots and 30 µg protein from leaves were loaded, and polyclonal antibodies raised against PvXDH protein were used.

3. PvXDH displays activity in presence of O_2 as the only electron acceptor and is able to produce ROS

Xanthine oxidoreductases (XOR) from animal and plant sources have been shown to transfer the electrons from the purine substrates, xanthine and hypoxanthine, to the final acceptor NAD^+, in the so called xanthine dehydrogenase (XDH) activity, but they can also transfer the electrons to O_2, acting as xanthine oxidase (XO) or NADH oxidase (NADHox), as depicted in Fig. 3A. In the XDH activity, oxidation of xanthine takes place at Moco center, and the electrons are rapidly transferred via the two [2Fe-2S] centers to FAD. Finally, at the FAD domain, the electrons from the reduced enzyme reach their final acceptor, NAD^+ (Fig. 3A, left). In the XO activity, electrons follow the same route, although O_2 instead of NAD^+ is the final electron acceptor (Fig. 3A, middle). In the NADHox activity, electrons go from NADH to O_2, involving the FAD domain of the protein, but not the Moco or the [2Fe-2S] clusters (Fig. 3A,

81

right).
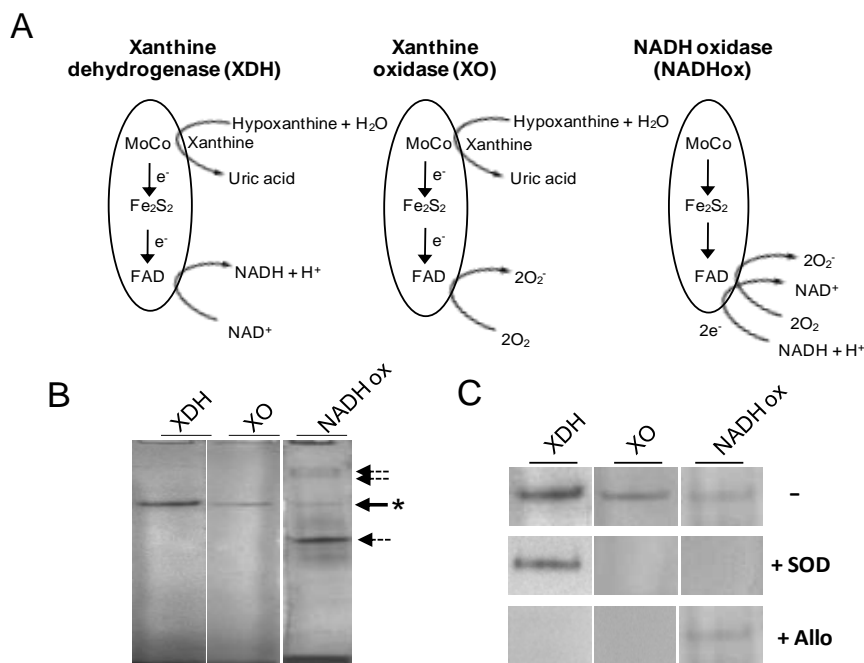
Fig. 3. Enzymatic activity of PvXDH. (A) Schematic representation of the enzymatic activity catalyzed by the protein. i) Xanthine deydrogenase activity (XDH) oxidizing xanthine to uric acid with NAD^+ as the final electron acceptor. ii) Xanthine oxidase activity (XO) oxidizing xanthine to uric acid with O_2 as electron acceptor. iii) NADH oxidase activity (NADHox) oxidizing NADH to NAD^+ with O_2 as electron acceptor. Arrows indicate electron flow over the domains. (B) In-gel assay of the activities XDH, XO and NADHox in leaf extracts from *P. vulgaris*. Arrow with asterisk indicates NADH oxidase activity of PvXDH enzyme. (C) XDH, XO and NADHox activities after the addition of 60 $U.mL^{-1}$ SOD (+ SOD) or 0.5 mM allopurinol (+ Allo). 50 μg of total soluble protein from leaf extracts were loaded in each lane and fractionated on PAGE under non-denaturing conditions. Representative results from at least 3 independent experiments are shown.

As is shown in Fig. 3B, XDH from *P. vulgaris* showed a predominant activity when hypoxanthine was used as electron donor and PMS served as the electron carrier intermediary catalyst. When PMS was not added, oxidase activities that co-migrated with the predominant XDH activity, were also detected, although these were much less efficients (about 20 and 8%, respectively), compared to XDH activity. When allopurinol, a XDH inhibitor, was added to the reaction mix, XDH and XO activities were abrogated, but not the NADHox activity, indicating its independence of the Moco domain (Fig. 3C). SOD did not affect PvXDH

activity when PMS served as the electron carrier catalyst (Fig. 3C). However, in the absence of PMS the reduction of NBT by hypoxanthine and by NADH (Fig. 3C) was totally inhibited by SOD, indicating that when O_2 acts as the only electron acceptor, PvXDH is able to produce O_2^-, using both, hypoxanthine or NADH as substrates.

4. PvXDH is up-regulated during drought stress

Plants of PHA-0683 (Tolerant, T), Great Northern (Intermediate, I) and PMB-0285 (Sensitive, S) common bean varieties, exhibiting different degree of drought tolerance (Riveiro, 2012) were subjected to water deficit during 7 days and the changes in expression and activity of PvXDH were analyzed in nodules, roots and leaves of the treated plants (Fig. 4). In nodules, mRNA levels of *PvXDH* remained constant in the tolerant and the intermediate varieties but showed a significant decrease in the most sensitive one (Fig. 4A). In roots, drought stress caused a reduction of XDH mRNA level in the tolerant and the intermediate genotypes. In contrast, in the most sensitive one the expression increased compared to the control roots. In leaves, similar increases of XDH mRNA level were observed in all varieties (Fig. 4A). Despite the inhibition of XDH expression in nodules of the sensitive genotype, only a slight decrease in XDH activity was found in drought-stressed nodules with respect to those from control plants, and there were no significant differences among any of the genotypes. Similarly, the effect of drought in XDH activity in the treated roots was different from that observed for *PvXDH* expression. XDH activity was kept at a constant level in the tolerant genotype and showed a moderate increment in the intermediate one. However, there was a high induction of XDH activity in drought-stressed roots of the sensitive plants, showing about a 3-fold increase respect to unstressed roots. Levels of XDH activity also increased in the drought-stressed leaves, although the changes were less pronounced than those observed in roots (Fig. 4B).
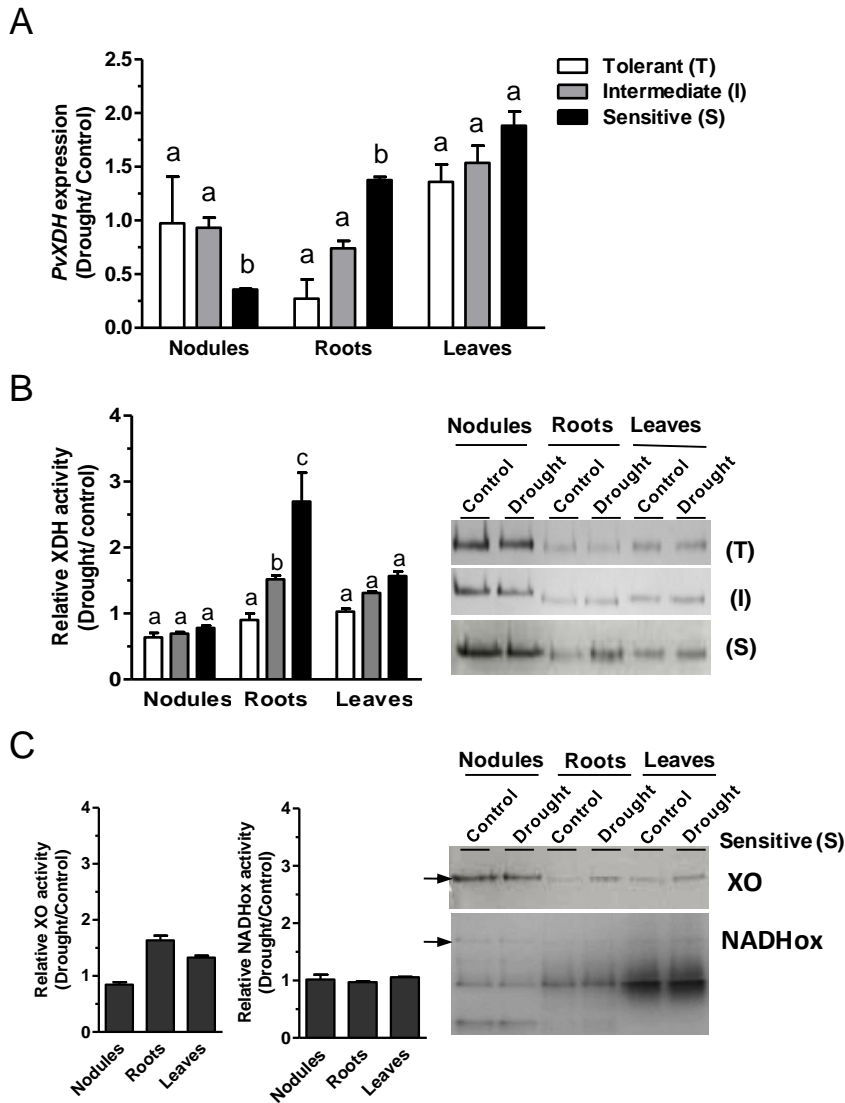
Fig. 4. PvXDH response to drought stress. (A) Relative XDH mRNA expression and (B) XDH activity in nodules, roots and leaves of tolerant (T), intermediate (I) and sensitive (S) common bean varieties after 7 days of water deprivation. (C) Xanthine oxidase (XO) and NADH oxidase (NADHox) activities in nodules, roots and leaves of the sensitive variety after 7 days of water deprivation (S). For activity assays, 5, 10 and 50 μg of total soluble protein from extracts from nodules, roots and leaves, respectively were loaded. Expression data were normalized according to *Actin-2* gene expression level. Data are shown as relative to the expression or activity in the control, unstressed samples. Quantitative analyses were made using Quantity One software (Bio-Rad). Results are representative of at least three independent experiments.

The effect of water deficit in the XO and NADHox activities of the enzyme was also checked in the most sensitive genotype in which mRNA expression showed the highest changes. As shown in Fig. 4C, there was only a slight increase of the

XO activity in the roots of the treated plants, while there were no significant changes in either the XO activity in nodules or leaves, nor in the NADHox activity in the tissues of the most sensitive genotype.

5. PvXDH is regulated by stress-related molecules

The *in silico* analysis, using the PlantCARE prediction software (Lescot *et al.,* 2002), of about 1.6 kb sequence upstream of the ATG codon of the *PvXDH* gene revealed the presence of multiple motifs related to plant responses to abiotic and biotic stresses (Fig. 5A). In addition, the proximal core promoter elements TATA-box and CAAT-box were found at 131 and 136 bp upstream of the transcription start site (Supplementary Fig. S3). Among the stress-related motifs, two abscisic acid-responsible (ABRE), one methyl jasmonate (CGTCA, TGACG) and several anaerobiosis-related (ARE) were identified. They were also two gibberellins (GARE or TATC-box), as well as others associated with low temperature (LTR), high temperature (HSE) and with response to fungal elicitors (Box-W1) motifs. Besides, the prediction also found several regulatory motifs associated with response to light within the analyzed sequence (not shown). Comparison of the *PvXDH* proximal promoter with similar regions from the promoter sequences of the two *AtXDH* genes showed shared, as well as distinct, regulatory sequences related to response to stresses among the three promoters. Abundance of stress-related regulatory motifs was higher in the *AtXDH1* promoter than in the *AtXDH2*, according to the reported stress-related induction of *AtXDH1* and the constitutive expression of *AtXDH2* (Hesberg *et al.,* 2004).

As a preliminary evaluation of the functionality of these *cis*-regulatory motifs, *PvXDH* gene expression and activity in response to stress conditions or stress-related treatments was determined. As shown in Fig. 5B, treatments with ABA, JA, SA, ACC, GA and SNP produced a slight induction of *PvXDH* mRNA expression. In contrast to the moderate effect in expression, most treatments exerted a significant induction of enzyme activity, whereas SNP was the only treatment that inhibited the XDH activity (Fig. 5D).
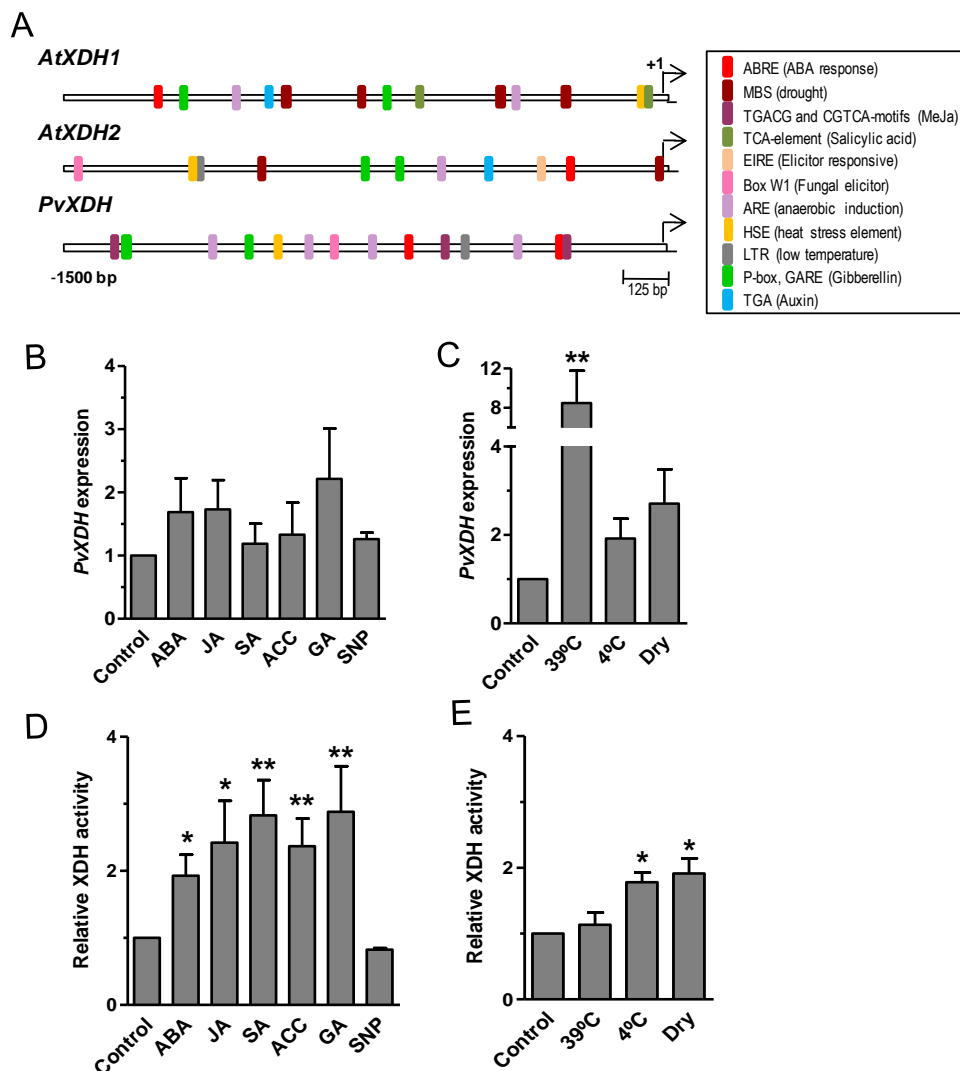
Fig. 5. PvXDH response to stress-related treatments. (A) Schematic representation of *cis*-regulatory elements in the promoter sequences of XDHs from *P. vulgaris* (*PvXDH*) and *A. thaliana* (*AtXDH1* and *AtXDH2*). (B) Relative XDH mRNA expression in leaves from *P. vulgaris* treated with ABA, JA, SA, ACC, GA and SNP. (C) Relative XDH mRNA expression in leaves from *P. vulgaris* submitted to high (39 °C) and low (4 °C) temperatures and after dehydration (Dry). (D) XDH activity in leaves after ABA, JA, SA, ACC, GA and SNP treatments. (E) XDH activity in leaves submitted to high (39 °C) and low (4 °C) temperatures and dehydration (Dry). Expression data were normalized to *Actin-2* gene expression. For XDH activity assay, 40-50 μg total soluble protein from crude extracts from leaves were loaded. Quantitative analyses were made using Quantity One software (Bio-Rad). Data are presented as relative to expression or activity in control samples. Gels show representative results of at least three independent experiments. Significant differences according to Student's t-test are denoted by asterisks (* *p*< 0.05, ** *p*< 0.01).

To study the response of PvXDH to temperature and dehydration stresses, detached leaves were subjected to either low (4 ºC) or high (39 ºC) temperatures during 4 and 2 hours, respectively, or desiccated until their water content was below 50% of control leaves. Heat stress caused a strong accumulation of *PvXDH* transcripts (approximately 8-fold), whereas cold stress and dehydration exerted only a slight induction of expression (Fig. 5C). However, cold stress and dehydration induced a significant increase of PvXDH activity whereas, despite the high effect on the gene expression, increments of activity were not observed in response to heat stress (Fig. 5E).

6. Nitric oxide inhibits PvXDH irreversibly *in vitro*

As previously shown in Fig. 5, XDH activity in leaves from plants treated with the nitric oxide (NO)-releasing compound SNP was slightly inhibited while the transcript abundance was slightly enhanced, thus suggesting posttranscriptional regulation of PvXDH protein mediated by NO. The i*n silico* analysis using several prediction platforms revealed several cysteine residues that could be *S*-nitrosylated as well as two possible tyrosine residues that could suffer nitration within the PvXDH amino acid sequence (Supplementary Fig. S1 and Supplementary Table S2). Moreover, the presence of metal centers in the functional motifs of PvXDH indicated that other modifications, as the nitrosylation of transition metals could also modulate XDH activity. To further investigate the NO effect on XDH protein, *in vitro* treatments of crude extracts were performed using NO-donors that release NO in different redox forms: GSNO and SNAP that produce NO radical (NO$^{\cdot}$) and SNP that releases the nitrosonium ion (NO^{+}). As is shown in Fig. 6A only SNP inhibited XDH activity when leaf extracts were incubated with 0.1 mM of each donor. Since the efficiency of NO released by these compounds may be affected by the experimental conditions, higher concentrations of GSNO and SNAP were used to check their inhibitory effects on XDH activity. When these chemicals were used at 0.5 and 1 mM, both compounds were able to inhibit PvXDH activity (Fig. 6B),

although activity level remained higher than when 0.1 mM of SNP was used. Potassium ferricyanide (FeCy), an analogue of SNP that is unable to release NO, also inhibited PvXDH activity, but contrary to the inactivation performed by SNP, the inhibition by FeCy was reversed by DTT (Fig. 6C), thus indicating different inhibitory mechanisms of these compounds. Inhibitory effect of SNP was kept after the inhibitor was removed by dialysis of the treated samples (not shown), thus suggesting that the enzyme was inhibited by covalent modification. Moreover, SNP inhibition of XDH activity did not take place when leaf extracts were dialyzed before the addition of SNP (Fig. 6D). Since ferrous iron present in extracts may enhance protein modifications mediated by NO, dialyzed leaf extracts were incubated with SNP and several concentrations of $FeSO_4$ (0.01, 0.1 and 1 mM). As shown in Fig. 6E, PvXDH activity was inhibited by increasing concentrations of added iron, even in the absence of SNP. When the dialyzed extracts were incubated with $FeSO_4$ plus SNP, the inhibition observed was higher than such showed when only iron was present. Thus, it seems that NO released by SNP is not able to act on the enzyme in absence of low molecular compounds, such as iron or some other molecules present in the crude extracts. Moreover, it has been reported that the effect of SNP may depend on the reduction state of the enzyme mediated by hypoxanthine (Ichimori *et al.* 1999). However, incubation of dialyzed leaf extracts mediated by hypoxanthine produced only a moderate decrease in XDH activity and the inhibitiont did not increase when SNP was added (Fig. 6F), thus indicating that reduction of the enzyme by hypoxanthine did not affect the SNP inhibition of PvXDH. Determination of XO and NADHox activities in leaf extracts treated with SNP showed that the XO but not the NADHox activity was inhibited by SNP, in a similar degree than the XDH activity (Fig. 6G-I).
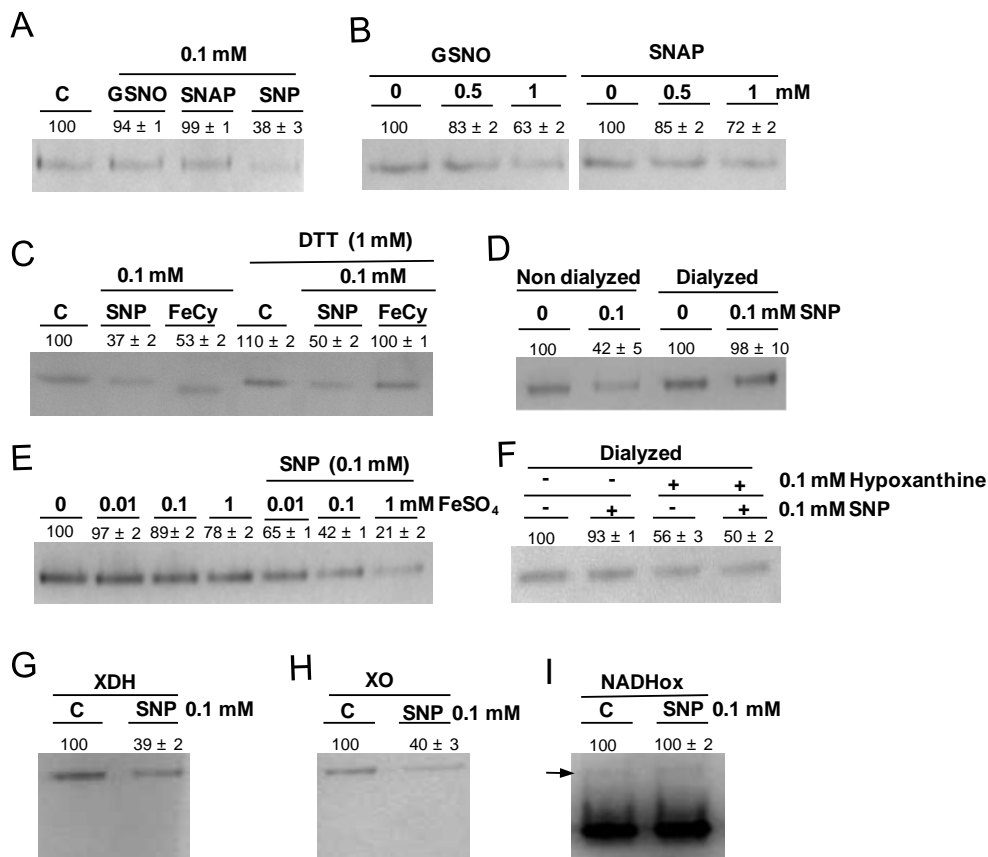
Fig. 6. *In vitro* **response of leaf PvXDH activity to NO-donors.** Relative XDH activity in (A) leaf extracts from *P. vulgaris* incubated with 0.1 mM of the NO-donors SNP, GSNO and SNAP; (B) leaf extracts incubated with 0.5 or 1 mM of GSNO and SNAP and (C) leaf extracts incubated with 0.1 mM of both SNP and potassium ferricyanide (FeCy), and after the addition of 10 mM DTT. (D) Relative PvXDH activity in leaf extracts non-dialyzed or dialyzed and treated with 0.1 mM SNP. (E) Relative PvXDH activity in leaf extracts dialyzed and treated with several concentrations (0, 0.01, 0.1 and 1 mM) of $FeSO_4$, or $FeSO_4$ plus 0.1 mM SNP. (F) PvXDH activity in leaf extracts dialyzed and treated with 0.1 mM SNP, 0.1 mM hypoxanthine or 0.1 mM hypoxanthine plus 0.1 mM SNP. (G) XDH, XO and NADHox activities in leaf extracts treated with 0.1 mM of SNP. Approximately 40-50 μg of total soluble protein from leaves were loaded in each line. Quantitative analyses were made using Quantity One software (Bio-Rad). Each figure represents one of at least 3 independent experiments that gave similar results.

7. Uric acid protects PvXDH from inhibition by NO

The effect of SNP on PvXDH activity was also examined in nodule extracts both *in vitro* and *in vivo* (Fig. 7). *In vitro* inhibition of PvXDH activity from nodule extracts required concentrations of SNP above 0.5 mM (Fig. 7A), thus suggesting the presence of compounds able to protect against SNP in these organs. Uric acid,

an abundant molecule in nodules of ureidic legumes can act as efficient scavenger of NO-derived peroxynitrite in animal and plant systems (Ames *et al.,* 1981; Kaur and Halliwell, 1990; Hilliker *et al.,* 1992; Alamillo and García-Olmedo, 2001). To examine whether uric acid contributes to reduce PvXDH susceptibility to NO, the capacity of SNP to inhibit XDH activity was tested in leaf extracts incubated with 0.5 µM uric acid. As is shown in Fig. 7B, the addition of uric acid totally prevented leaf PvXDH from inhibition by SNP, suggesting that the levels of uric acid in nodules may help to protect XDH against NO deleterious effects. To study the *in vivo* relevance of PvXDH inhibition by SNP, nodulated plants were irrigated with nitrogen-free solution containing 0.5 or 1 mM of SNP for 2 days. Following SNP application, XDH activity was not affected by the SNP treatment at any of the concentrations used (Fig. 7C). Accordingly, SNP did not exert any negative effect in nodule function, since nitrogenase activity was similar in the treated and control plants (Fig. 7D).

To further test whether uric acid may exert a protective role against NO radicals *in vivo*, we analyzed the effect of SNP in transgenic hairy roots in which the expression of *PvXDH* gene has been partially inhibited by RNAi (Fig. 8A). As expected, the lower abundance of *PvXDH* expression was followed by a lower level of XDH activity in RNAi-silenced roots and in the nodules derived from them (Fig. 8B and 8C). Since uric acid is the primary product of XDH activity, the silenced roots and nodules are expected to contain lower levels of this compound. When roots extracts from the RNAi-*PvXDH* plants were treated with SNP, we observed that XDH activity was inhibited by about 60%, both in the roots transformed with the empty vector and in the RNAi-*PvXDH* roots. Moreover, the levels of inhibition were similar to those observed in SNP treated leaf extracts (Fig. 8D). In contrast, a significant higher inhibition by SNP was found in the treated nodules from the RNAi-*PvXDH* roots than in those from hairy roots transformed with the empty vector used as controls, thus indicating that uric acid could prevent the NO inhibitory effect (Fig. 8D).
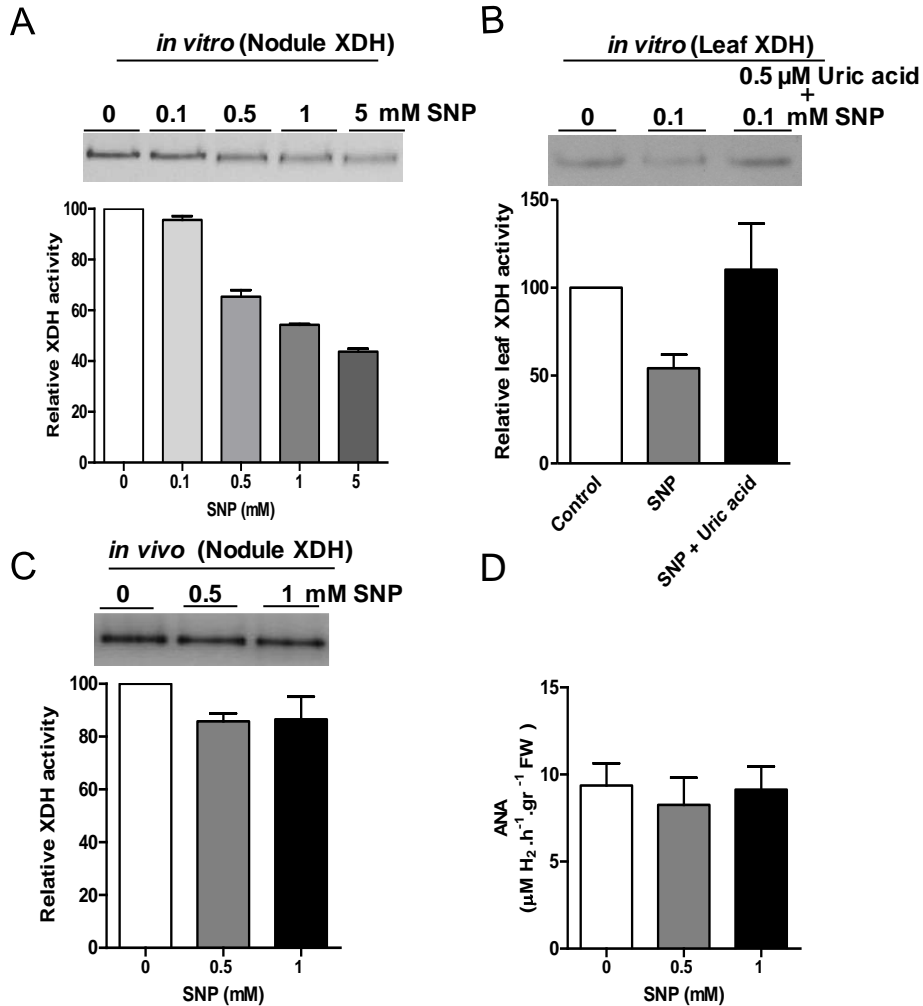
Fig. 7. Effect of SNP in nodules from *P. vulgaris*. (A) Relative XDH activity in nodule extracts from *P. vulgaris* treated with increasing concentrations (0.1, 0.5, 1 and 5 mM) of SNP. (B) Relative PvXDH activity in leaf extracts treated with 0.1 mM SNP and 0.1 mM SNP plus 0.5 μM uric acid. (C) Relative XDH activity in nodules extracts and (D) BNF measured as apparent nitrogenase activity (ANA) of plants irrigated with the nutrient solution containing 0.5 or 1 mM SNP. For activity assays, 5 and 50 μg of total soluble protein from nodules and leaves, respectively, were loaded in each line. Quantitative analyses were made using Quantity One software (Bio-Rad). Each figure represents one representative result from at least 3 independent experiments.
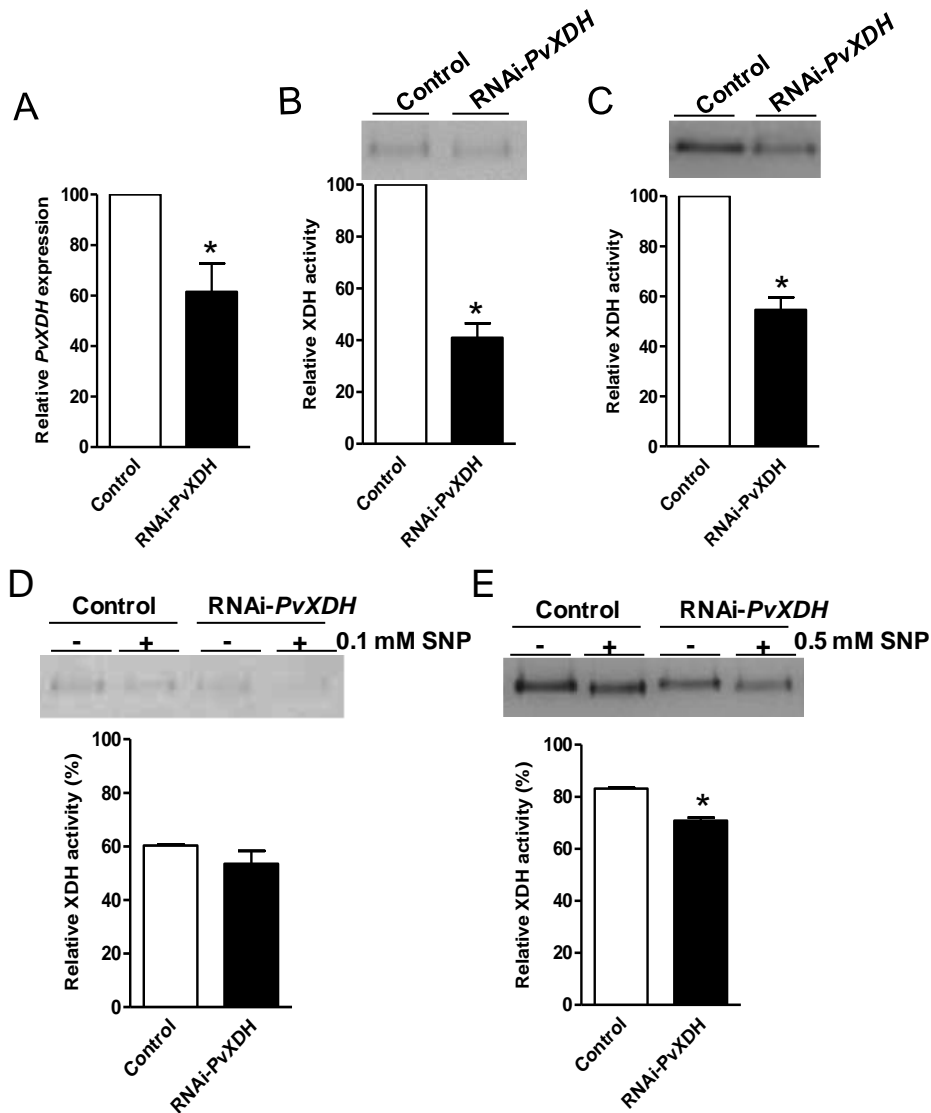
Fig. 8. Effect of SNP treatment in *PvXDH* mRNA silenced tissues. (A) Relative XDH mRNA expression in RNAi-*PvXDH* and empty vector (control) transformed hairy roots from *P. vulgaris*. (B) Relative PvXDH activity in transformed hairy roots and (C) in nodules derived from transformed hairy roots. (D and E) Percentage of PvXDH activity in RNAi-*PvXDH* hairy roots and nodules after treatment of crude extracts with SNP and relative to non treated crude extracts. For activity assays 5 and 7.5 μg of total soluble protein from nodules and roots, respectively, were loaded in each line. Quantitative analyses were made using Quantity One software (Bio-Rad). Gels show representative results from at least 3 independent experiments. Significant differences according to Student´s t-test are denoted by asterisks (* $p<0.05$).

Discussion

Xanthine oxidoreductase (XOR) is an essential enzyme in purine catabolism pathway in all organisms, where it catalyzes the oxidation of hypoxanthine and xanthine to uric acid. Besides this housekeeping role, XOR has been implicated in responses to stress both in animals and plants. In mammals, the implication of XOR in stress is mainly due to its activity as xanthine oxidase (XO) which, unlike xanthine dehydrogenase (XDH) activity, can only use O_2 as the acceptor of the electrons released in the oxidation of xanthine/hypoxanthine, thus releasing O_2^- and H_2O_2 (Harrison, 2002). Moreover, ROS could also be released by the intrinsic NADH oxidase (NADHox) activity of the enzyme (Sanders *et al.,* 1996). The biochemical and molecular analyses performed so far indicate that XORs from plant sources appear only in the XDH form under physiological conditions (Boland, 1981; Pérez-Vicente *et al.,* 1992; Montalbini, 1998, 2000; Sauer, *et al.* 2002; Hesberg *et al.,* 2004), which preferentially uses NAD^+ as electron acceptor. In the present work, the only sequence coding for XDH in *Phaseolus vulgaris* genome has been isolated and characterized. The amino acid sequence showed the typical three domains structure of XORs (Fig. 1), and a high degree of similarity with the *AtXDH1* and *AtXDH2* proteins from *Arabidopsis thaliana* and with the human XDH (Supplementary Fig. S1). Alike XDHs from Arabidopsis (Hesberg *et al.,* 2004), the protein did not show the cysteine residues known to be responsible of the reversible conversion of mammalian XDH to XO (Nishino and Nishino, 1997; Nishino *et al.,* 2005) (Supplementary Fig. S1), further supporting that the plant enzyme should exist only in its XDH form. However, the function of XORs from some plant species has been also related with the production of ROS. Several works have shown induction of expression and activity of XDH by stress or physiological conditions that involve the generation of ROS (Montalbini, 1992a, 1995; Pastori and Del Río, 1997; Hesberg *et al.,* 2004; Yesbergenova *et al.,* 2005; Watanabe *et al.,* 2010). Moreover, ROS producing activities have been observed *in vitro* and *in vivo* in Arabidopsis and tomato XDHs (Hesberg *et al.,*

2004; Yesbergenova *et al.,* 2005; Zarepour *et al.,* 2010). However, the actual contribution of XDH activity to ROS production *in vivo* is still unclear, mainly due to the high concentration of NAD^+ in plant cells and the preference of the enzyme for this substrate (Hille and Nishino, 1995).

We have measured the XDH, XO and NADHox activities in crude extracts from leaves from *P. vulgaris* (Fig. 3B). The three activities appeared to correspond to XOR enzyme, as indicated by the appearance of formazan bands at the same position in the gel. Moreover, the complete inhibition of XDH and XO activities by the typical XDH inhibitor allopurinol (Fig. 3C) further indicated that the protein bands detected in all these enzymatic assays corresponded to the XOR protein. PvXDH was able to use O_2 as the only electron acceptor *in vitro* functioning as a XO or NADHox (Fig. 3B) and these activities released O_2^-, as demonstrated by the complete abolishment of NBT reduction by SOD (Fig. 3C). The XO and NADHox activities were lower than XDH activity (Fig. 3B), and thus, it is very likely that they would have little functional relevance *in vivo* in *P. vulgaris*.

In ureidic legumes as common bean, XDH plays an important role in primary nitrogen metabolism, since the product of XDH reaction, uric acid, is further oxidized to the ureides allantoin and allantoate, which are the molecules that transport the fixed nitrogen from the nodules to the whole plant (Stasolla *et al.,* 2003; Zrenner *et al.,* 2006). According to this function, the expression and enzymatic activity of PvXDH was largely enhanced in nodules, compared to other plant tissues (Fig. 2). Apart from this essential role in primary nitrogen metabolism, there are reports indicating that an activation of purine catabolism pathway occurs during some stress conditions in ureidic legumes (Alamillo *et al.,* 2010; Gil-Quintana *et al.,* 2013). We analyzed PvXDH levels in several common bean varieties with different tolerance to water stress (Riveiro, 2012) that show accumulation of ureides in the leaves according to the degree of sensitivity to drought (Chapter 2). In addition, nodules of stressed plants became senescent soon after drought exposure and thus N_2 fixation is impaired before the

accumulation of ureides in aerial tissues. Therefore, the ureides accumulated upon drought stress were not originated from enhanced incorporation of fixed nitrogen to the *de novo* synthesis of purine nucleotides (Alamillo *et al.,* 2010; Chapter 2, Fig. 1). These findings led to propose that alternative pathways of ureide production are activated in aerial tissues from common bean (Alamillo *et al.,* 2010). Results presented here agree with this hypothesis since XDH in nodules of drought-stressed plants declined rapidly after treatment but it increased in other plant tissues (Fig. 4A and 4B). In roots, the enhancement of expression and activity of PvXDH correlated with the ureide level of each genotype (Fig. 4A and 4B; Chapter 2, Fig. 3), thus indicating that purine catabolism was activated in each variety in a degree that correlated to its sensitivity to the stress. The induction of PvXDH activity was more pronounced in roots of stressed plants, where there were higher levels of PvXDH than in leaves (Fig. 2). Drought is one of the abiotic stresses causing major impact in redox homeostasis (Golldack *et al.,* 2014), therefore, PvXDH activity might be contributing to ROS generation during this situation. Contrary to this hypothesis, only slight changes occurred in the XO activity and no changes were observed for the NADHox activity in the stressed tissues of the most sensitive genotype (Fig. 4C), supporting the idea that ureide production but not ROS production is the main function accomplished by XDH protein in ureidic legumes.

The moderate increase of *PvXDH* transcription levels under stress conditions or stress-related treatments (Fig. 5B and 5C) provides preliminary evidence that the regulatory motifs found in the proximal promoter sequence of *PvXDH* are functionally active. However, in almost all cases, the effects in activity were more pronounced that such of mRNA expression (Fig. 5D and 5E), thus suggesting post-translational regulation for the PvXDH enzyme. It is well known that XDH requires a post-translational activation of the holoenzyme by the Moco sulfurase ABA3. Moreover, Moco sulfurase ABA3 controls the activity of XDH according to environmental conditions (Bittner *et al.,* 2001), so that the ratio of sulfurated (active) to non-sulfurated (inactive) enzyme is relatively independent from the

total amount of holoprotein (Hesberg *et al.,* 2004).

However, it is also possible that the low effects found in the levels of expression were due to the delay in sample analysis, since the treated tissues were collected 48 hours after treatments, time in which promoter activation probably had already declined.

In the treatment of high temperature, XDH activity was not induced, in spite of the dramatic increase of expression observed (Fig. 5C and 5E). Likely, low stability of the enzyme under this situation may require high rates of transcript expression in order to ensure enough active XDH to fulfill its housekeeping functions. Increased XDH activity in plants has been associated with natural senescence (Pastori and Del Río, 1997) and with physiological stressful processes, such as pathogen defense (Montalbini, 1992a), hypersensitive cell death (Montalbini, 1995) and responses to drought (Yesbergenova *et al.,* 2005; Watanabe *et al.,* 2010;). Signaling molecules used in this study, such as abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), ethylene (ACC) and gibberellins (GA) have been involved in complex interconnected pathways that control gene expression in biotic and abiotic stress responses, as well as during natural senescence. On the other hand, it is known that, at the molecular level, a crosstalk exists between senescence-associated and environmentally-induced cell death, whose final common purpose is to ensure the remobilization of nutrients for their transport to other parts of the plant (Feller and Fischer, 1994; Hörtensteiner and Feller, 2002). The central role of XDH in purine catabolism and the changes in gene expression and activity in the studied conditions strongly supports a potential function of PvXDH in nitrogen remobilization to sustain plant growth under stress conditions. The higher similarity of *PvXDH* promoter structure with that from *AtXDH1*, which has been shown to be induced by abiotic stress, in comparison with that from *AtXDH2*, whose constitutive low expression level has been reported (Hesberg *et al.* 2004), also supports this idea. Nakagawa *et al.* 2007 observed that in Arabidopsis *XDH*-suppressed lines, vegetative and reproductive growth were impaired and plants showed early senescence,

indicating the requirement of XDH to achieve normal development of the plants. It is very likely that the observed increase in *PvXDH* expression and activity occurs as a result of a general enhancement of ROS promoted by the physiological stress situations to which plants have been subjected in this work, rather than to serve as a source of them. Additionally, the ureides that probably are accumulating during all these situations in common bean, as occurs during drought, also may serve as a ROS scavengers (Brychkova *et al.,* 2008; Alamillo *et al.,* 2010).

Interestingly, the only treatment that did not lead to the induction of PvXDH activity was the addition of the NO-releasing compound SNP (Fig. 5B). NO is a signal molecule implicated in plant senescence and environmental stress (Prochazkova and Wilhelmova, 2011). Moreover, as a molybdoenzyme, XOR have been suggested as a possible source for NO synthesis through the reduction of nitrite to NO (Gupta *et al.,* 2011; Cantu-Medellin and Kelley, 2013). However, it looks contradictory that plant XDH could be generating NO *in vivo*, in particular, under up-regulating stress conditions, while NO is inhibiting the enzyme. A possible explanation for this paradox is that increments in expression levels under most stress conditions are probably high enough to overcome the limitation imposed by NO inhibition of activity, therefore ensuring synthesis of ureides that could be used for nitrogen recycling and *in vivo* protection against the NO effect.

There are three major protein posttranslational modifications by which NO can affect protein structure and function, covalent nitration of tyrosine residues (to form 3-nitrotyrosine), modification of thiols (S-nitrosylation), and NO binding to metal centers (metal nitrosylation) (Gow *et al.,* 2004; Gupta, 2011; Astier and Lindermayr, 2012; Corpas *et al.,* 2013; Kovacs and Lindermayr, 2013; Lamotte *et al.,* 2014). From these, reversible S-nitrosylation is probably the one with largest contribution of protein functional regulation both under physiological and stress conditions, whereas nitration of tyrosines usually results in protein inactivation, mainly under stress. *In silico* predictions have suggested that several cysteine

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residues may be modified by S-nitrosylation in PvXDH (Supplementary Fig. S1 and Supplementary Table S2). S-nitrosoglutathione (GSNO) and S-nitroso-N-acetyl-DL-penicillamine (SNAP) are nitrosylating agents which mediate the post-translational process of S-nitrosylation on proteins resulting in modulation of their activity (Lamotte *et al.,* 2014). However, incubation of leaf extracts from *P. vulgaris* with these two NO donors resulted only in a slight inhibition of XDH activity (Fig. 6A). Although the efficiency of the NO release is very different among different NO donors (Ederli *et al.*, 2009), the 5-fold higher concentration of GSNO required for the inhibition of PvXDH activity (Fig. 6B), strongly suggests that S-nitrosylation is not the mechanism by which NO inhibits the enzyme. Moreover, DTT, which is able to reduce nitroso (S–NO) adducts in protein thiol groups, did not revert the *in vitro* inhibition of PvXDH by SNP (Fig. 6C), further supporting the same conclusion.

The main nitrating agent *in vivo* is peroxynitrite ($ONOO^-$) which is formed rapidly in the reaction of the superoxide anion (O_2^-) with NO (Abello *et al.,* 2009; Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011). Transition metals, such as iron in his ferrous form (Fe^{+2}) have been shown to take part in protein Tyr nitration process by $ONOO^-$ *in vitro* (Campolo *et al.,* 2014). Probably, Fe^{+2} added to the extracts contributed to $ONOO^-$ formation increasing the production of O_2^- by the Fenton reaction, in which Fe^{+2} and O_2 generate Fe^{+3} and O_2^-. The irreversible character of the inhibition observed and the Fe^{+2} requirement for such inhibition allows us to propose Tyr-nitration as the most likely modification that is modulating PvXDH activity, although further research with the purified enzyme need to be done to confirm this hypothesis. Previous studies showed an *in vitro* and *in vivo* inhibition of the mammalian XDH activity by NO (Hassoun *et al.,* 1995; Ichimori *et al.,* 1999). Ichimori *et al.* (1999), showed that XDH and XO from animal sources were susceptible to NO inhibition only when they were reduced by their substrate, hypoxanthine. These authors proposed that the inactivation of the enzyme occurred through the reaction of NO with and essential sulfur of the reduced molybdenum center.

Our results suggest that PvXDH could be affected by SNP also at the molybdenum cofactor domain, since only XDH and XO activities, that need the Moco domain to bind de substrate hypoxanthine or xanthine, were inhibited, whereas the NADHox activity, requiring only the FAD domain, was not affected by SNP (Fig. 6G). Using the SNP analogue FeCy, which is known to be able to oxidize XDH (Pérez-Vicente *et al.,* 1988), we have further demonstrated that this compound inhibits PvXDH activity by a mechanism that, in contrast to the SNP-mediated inhibition, can be reverted by reducing agents (Fig. 6C), and therefore, the effect of SNP could not be merely due to the oxidation of susceptible residues in the enzyme.

It is noteworthy that the concentration of SNP capable of the *in vitro* inhibition of PvXDH activity in extracts from leaves is not enough to perform the inhibition in nodule extracts, where at least 5-fold more SNP was needed to perform a similar degree of inhibition (Fig. 7A). Nodules are organs with an elevated metabolic activity that contains high concentrations of antioxidants to prevent ROS/RNS damage (Becana *et al.,* 2000; Matamoros *et al.,* 2003). In particular, leghemoglobin, which is present at concentrations of 1-5 mM in nodules, is found to bind NO with high avidity (Meakin *et al.,* 2007; Navascues *et al.,* 2012) thus limiting the NO inhibitory effects. Moreover, several works have shown that uric acid can act as antioxidant molecule in animal and plant systems (Ames *et al.,* 1981; Kaur and Halliwell, 1990; Hilliker *et al.,* 1992). Uric acid has been recognized as a natural scavenger of $ONOO^-$, selectively binding and inactivating it (Whiteman and Halliwell, 1996; Alamillo and García-Olmedo, 2001). Results presented in Fig. 7 indicated that uric acid, produced at large concentrations in nodules of ureidic legumes by the high XDH activity in these tissues, does protect XDH from the inhibitory effect of NO. As only ureides and not uric acid are supposed to be exported, due to the quick oxidation of uric acid by the high uricase activity found in nodules (Sánchez *et al.,* 1987), the concentration of this compound is expected to be significantly lower in leaves than in nodules. This fact, together to the leghemoglobin protective effect, could explain the different

level of inhibition by SNP among these tissues and the blockade of the inhibition exerted by exogenous uric acid added to the SNP-treated leaf extracts (Fig. 7).

Inhibition of *PvXDH* gene expression in transgenic roots leads to a significant reduction in the XDH activity in the silenced tissues and in the nodules developed in these roots (Fig. 8B and 8C). Therefore, a consequent decrease in the concentration of uric acid, which is a product of this enzyme, may be occurring in the silenced nodules. In agreement, the degree of PvXDH inhibition by 0.5 mM SNP in nodule extracts from transgenic silenced roots was significantly higher than in nodules from control transgenic roots (Fig. 8E). Results from Fig 7B and 8 suggest that uric acid may help to protect XDH protein from the deleterious effect of NO in the nodules from this legume. NO produced from the reduction of nitrate and nitrite by nitrate reductase has been related with the inhibition of nitrogenase activity in amidic legumes as Lotus and Medicago (Kato *et al.,* 2010; Melo *et al.,* 2011). However, the *in vivo* application of SNP to nodulated plants of *P. vulgaris* did not lead to the inhibition of neither XDH activity nor nitrogenase activity (Fig. 7C and 7D). Interestingly, the inhibition of N_2 fixation by nitrate has been also reported in ureidic legumes as soybean (Kanayama and Yamamoto, 1990). Moreover, in a recent work from our group, we have observed that the irrigation of nodulated plants of *P. vulgaris* with 10 mM nitrate lead to the total abolishment of N_2 fixation after 2 days of treatment. Nevertheless, results presented here suggest that the inhibition of N_2 fixation by nitrate should be exerted by a NO-independent mechanism in ureidic legumes.

In summary, we show that xanthine dehydrogenase from *P. vulgaris* is expressed to higher levels in nodules than in any other tissue, and that the enzyme is regulated both at transcriptional and posttranscriptional levels under stress conditions. Analysis of the inhibitory effect of NO on the enzyme activity suggest that NO, in particular NO^+ released by SNP, does inhibits the enzyme *in vitro*, probably through nitration of Tyr residues or by interaction with metal centers. However, the enzyme is protected from this deleterious effect in nodules, probably by the high levels of its own reaction product, uric acid.

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Supplementary material

Supplementary Fig. S1. Organization of XDH protein from *Phaseolus vulgaris* showing predicted posttranslational modifications.

Supplementary Fig. S2. Linearity of the xanthine dehydrogenase (XDH) assay.

Supplementary Fig. S3. Identification of putative *cis*-regulatory motifs in the proximal promoter sequences of the *PvXDH* gene

Supplementary Table S1. List of primers used in this study.

Supplementary Table S2. S-nitrosylation and Tyr-nitration sites predicted in PvXDH protein.

A

B

Supplementary Fig. S1. Organization of XDH protein from *Phaseolus vulgaris* showing predicted posttranslational modifications. (A) Draft of the XDH protein sequence from *Phaseolus vulgaris* showing predicted posttranslational modifications. Phosphorylations (Pho) at positions 52, 254 and 354 and N-glycosilation (N-Gly) at position 1103 are shown in comparison with the predicted for human XDH http://www.phosphosite.org. Tyrosine residues of PvXDH susceptible to be nitrated (Y-NO$_2$), predicted with GPS-YNO2 1.0 software, are shown at positions 23 and

490. Cysteine residues susceptibles to be S-nitrosylated (S-NO), predicted with GPS-SNO 1.0 software, are shown at positions 77, 357, 689, 1124 and 1342. (B) Alignment of the deduced amino acid sequence of XDH from *P. vulgaris* with XDHs from human and Arabidopsis. Sequences were obtained from the NCBI database (accession numbers are shown in brackets). *H. sapiens* (XP_011531397), *A. thaliana* XDH1 (NP_195215) and *A. thaliana* XDH2 (NP_195216). Functional domains: N-terminal iron-sulfur clusters [2Fe-2S], FAD binding domain and molybdenum cofactor binding domain are emphasized by shading. Conserved cysteines ligating each [2Fe-2S] cluster are boxed. FFLPGYR motif supposed to be responsible for binding NAD$^+$ (Amaya *et al*., 1990) to FAD binding domain is underlined. Conserved Glu-832 and Arg-910 residues supposed to be essentials for binding and proper positioning of purine substrates (Glatigny *et al*., 1998) in the molybdenum cofactor binding domain are boxed. Cysteine residues susceptibles to be oxidated (Nishino and Nishino, 1998) and peptide sequence susceptible to be removed by irreversible proteolysis in *H. sapiens* protein (Nishino *et al*., 2008) for the conversion of XDH into XO are denoted in red. Residues predicted to be nitrated, phosphorylated, S-nitrosylated and N-glycosilated are shown in bright green, red, green and purple, respectively. Phosphorylations and N-glycosilation on XDHs from *A. thaliana* and *P. vulgaris* are shown in comparison with *H. sapiens.* The sequences were aligned for maximum matching using the CLUSTAL W method.
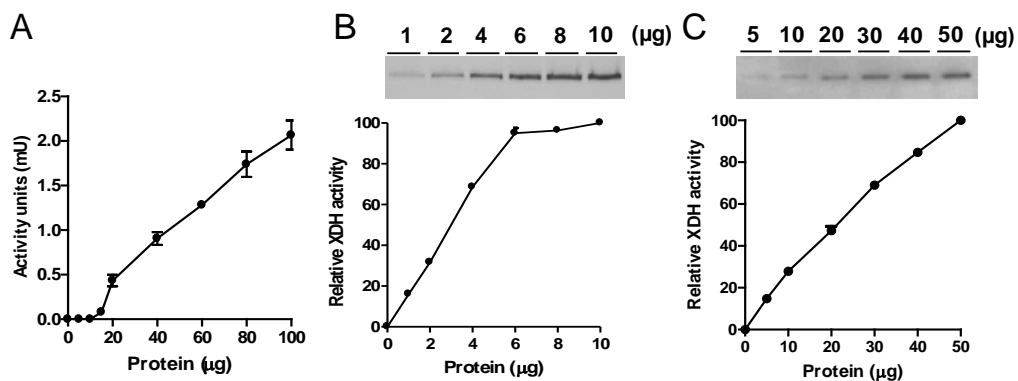
Supplementary Fig. S2. Linearity of the xanthine dehydrogenase (XDH) assays. (A) Linearity of the XDH spectrophotometric assay. XDH activity was determined by the reduction of NAD^+ to NADH at 340 nm using 1-100 µg of total protein from nodule crude extracts. (B) In-gel XDH activity assay in nodule crude extracts. The assay was linear using 1-6 µg of total protein. (C) In-gel XDH activity assay in leaf crude extracts. The assay was linear using 5-50 µg total protein.

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-1500 AAGATTCCATCATTCTTTTTATGCATTCAAACTCTTGAATATACTAGACTCACAATAATGCCTATTGATACTCCTAGCAA
      TTCTAAGGTAGTAAGAAAAATACGTAAGTTTGAGAACTTATATGATCTGAGTGTTATTACGGATAACTATGAGGATCGTT
                                                         GCTCA-motif (MeJa)
-1420 AAAAAAAACTACTACAGTTCCTTCGATTTTACACAGGTGAAGGTTCAAATTAAGCGTCAGAAAATGGTGGAACTTCCAGA
      TTTTTTTTGATGATGTCAAGGAAGCTAAAATGTGTCCACTTCCAAGTTTAATTCGCAGTCTTTTACCACCTTGAAGGTCT
        GARE-motif (Gibberellin)
-1340 AACAGACGATGGAATTGCACAATGGTGCAAAGACGCATTTGTTGCCAAGGTATCACAGATTTCTTAAATTAAGTTTTTCT
      TTGTCTGCTACCTTAACGTGTTACCACGTTTCTGCGTAAACAACGGTTCCATAGTGTCTAAAGAATTTAATTCAAAAAGA

-1260 TTGGCGCTTGTCATAGTTGCTTTTACAAAGGTTGTTTTACACATGTGCACAAACTTTTGTACGACTCAACTCAAATATCC
      AACCGCGAACAGTATCAACGAAAATGTTTCCAACAAAATGTGTACACGTGTTTGAAAACATGCTGAGTTGAGTTTATAGG

-1180 ATTCACAGATTCTTAAAGCTTTTGTATAATCTATGATAATGCTTGCTTTGTAAACCACCAGGATGCTTTGCTCGAGAAAT
      TAAGTGTCTAAGAATTTCGAAAACATATTAGATACTATTACGAACGAAACATTTGGTGGTCCTACGAAACGAGCTCTTTA
                                                          ARE (anaerobic)
-1100 ATAACACTACAGAAATATTCAGTGACCAAGAACTTCAACAAATTCATCGACCTAAAATATCTGTTTTGGTAAGATGCTAG
      TATTGTGATGTCTTTATAAGTCACTGGTTCTTGAAGTTGTTTAAGTAGCTGGATTTTATAGACAAAACCATTCTACGATC
                                                         GARE-motif (Gibberellin)
-1020 CACGACAGATACATGTGTGTTTTTTCACATGTAGTCGAGTTTCATTATATTTTTTCGATATATCACATGAAGATCGACCA
      GTGCTGTCTATGTACACACAAAAAAGTGTACATCAGCTCAAAGTAATATAAAAAAGCTATATAGTGTACTTCTAGCTGGT
                                                   HSE (Heat shock element) ARE (anaerobic)
-940  AACTAATTTGCCTAGATTTCTAAAAAAACTGCCATGTTTTGGTGTTTATGCAGGTGGTGGTTTGTTGGTTATGTCTTCTA
      TTGATTAAACGGATCTAAAGATTTTTTTGACGGTACAAAACCACAAATACGTCCACCACCAAACAACCAATACAGAAGAT
                                                   Box W1 (Fungal elicitor)
-860  GGGTTTCTTCTCTATAAATTCTTCCAATTGACCTCACTCCTCTCCTCGTGGATAGGCATCTTCTTAACAGTGCTTTTCTT
      CCCAAAGAAGAGATATTTAAGAAGGTTAACTGGAGTGAGGAGAGGAGCACCTATCCGTAGAAGAATTGTCACGAAAAGAA
                                                                ARE (anaerobic)
-780  GCTCCTGGTAACAGTGATCATGGAAATCTTAATACATTCGTCTCAATCAGAGCGTTCCAAATCCATGGTTTTACCCACAC
      CGAGGACCATTGTCACTAGTACCTTTAGAATTATGTAAGCAGAGTTAGTCTCGCAAGGTTTAGGTACCAAAATGGGTGTG
                                                                              ABRE
-700  AAGACCCCTTGAAGCAGAACCTTCTTCACCAATGATTCACCCATGTTTCTTAATGTTTATCACATTTCTTTCTCTCACAC
      TTCTGGGGAACTTCGTCTTGGAAGAAGTGGTTACTAAGTGGGTACAAAGAATTACAAATAGTGTAAAGAAAGAGAGTGTG
       (ABA)                                              GCTCA-motif (MeJa)
-620  GTGTATACCATTACTCTTGTTTATCTCTTTTTAATTACACGAATTAAGAAAAATAACGTCACATCACATCTATTCTCTGT
      CACATATGGTAATGAGAACAAATAGAGAAAAATTAATGTGCTTAATTCTTTTTATTGCAGTGTAGTGTAGATAAGAGACA

-540  TCCACCTCTGTTCTCGTCTTGTCTTTCTTTTTTCGGGTAGGAAACTTTTCATCTGTTAGGTTTGAAAATAAATTACCATA
      AGGTGGAGACAAGAGCAGAACAGAAAGAAAAAGCCCATCCTTTGAAAAGTAGACAATCCAAACTTTTATTTAATGGTAT
                                                 LTR (Low temperature regulation)
-460  TTTAATAAATAATTACATATATCCAAGCTATAAAGTTGAATATTTTTTTTCCGTTTTAGGACTTCAATAAATATCATAAA
      AAATTATTTATTAATGTATATAGGTTCGATATTTCAACTTATAAAAAAAAAGGCAAAATCCTGAAGTTATTTATAGTATTT
                ARE (anaerobic)
-380  CTGATTTTTTTAATGGTTTTAATAAAATGCAATGCATCTTTATGGTGTAGGAAGAAACGTTGGATAACCGCCTCAACTTTT
      GACTAAAAAAATTACCAAAATTATTTACGTTACGTAGAAATACCACATCCTTCTTTGCAACCTATTGGCGGAGTTGAAAA
                                                ABRE (ABA)  GCTCA-motif (MeJa)
-300  TCCACTGAAAAAAAAAAAAAAAAATAGTGTGTGATTTTGAATCCTACGTAGCAGAGACAGTGACGTTCCTATTCGCAACAAC
      AGGTGACTTTTTTTTTTTTTTTATCACACACTAAAACTTAGGATGCATCGTCTCTGTCACTGCAAGGATAAGCGTTGTTG

-220  GATAAGCCAATCGGCGTTATTCACTAGAAGATAAATCCGTTGCGCCAAAACTCTTTTCCACTTTTTTTAATTTTTCCTGT
      CTATTCGGTTAGCCGCAATAAGTGATCTTCTATTTAGGCAACGCGGTTTTGAGAAAAGGTGAAAAAAATTAAAAAGGACA

-140  TCAATTTTATAACCTTTTCTTCTCTGCAACGTTTCATTTGTTTATTCGTTTATTGCCTAATCGTCTTTCGTGGGTGAGTT
      AGTTAAAATATTGGAAAAGAAGAGACGTTGCAAAGTAAACAAATAAGCAAATAACGGATTAGCAGAAAGCACCCACTCAA
                                                                                →
-60   GGAGACACCTTTGAATCGCTGTTTATTTGTTTCGTTCTCATCAGAAGTGTGAAGTGGAGCTAACTGAGTGCTGTGAAAAA
      CCTCTGTGGAAACTTAGCGACAAATAAACAAAGCAAGAGTAGTCTTCACACTTCACCTCGATTGACTCACGACACTTTTT

      CCGTAGGAGTTTGTTAGAATCACTGAAAGAAGAAAGCTGCTCCGTTCCGTTCGTTTCCatg
      GGCATCCTCAAACAATCTTAGTGACTTTCTTCTTTCGACGAGGCAAGGCAAGCAAAGGtac
```
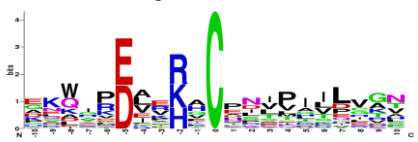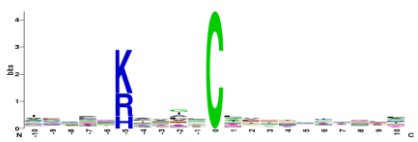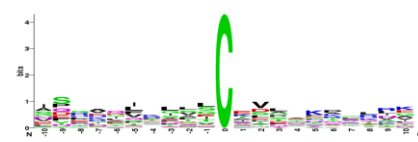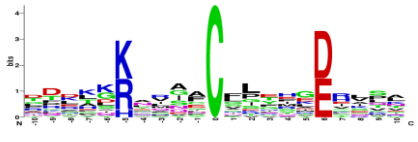
Supplementary Fig. S3. Identification of putative *cis*-regulatory motifs in the proximal promoter sequences of the *PvXDH* gene. 1500 bp up-stream of the transcription start (→) are shown. Shaded grey are the 5´UTR sequences. TATA-box and CAAT-box are underlined. *Cis*-regulatory motifs appear as colour-shaded. Promoter analysis was done using the PlantCARE software (Lescot *et al.* 2002).

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Supplementary Table S1. List of primers used in this study. Restriction sites of enzymes used for cloning are underlined.

Primer use	Gene	Primers sequences (5´- 3´)
XDH cDNA isolation	*PvXDH*	FWR (ATGGGATCGTTGAAGACGGA)
		REV (TCAAACACTAAGTTTGGGATGGA)
Promoter and 5´-UTR isolation	*PvXDH*	GSP1 (ACATGCATCCCTTCTACAGAATATAG)
		GSP2 (TCCATTAACATACACGATAGCCTCA)
qRT-PCR	*PvAct-2* (KF033666.1)	FWR (GGAGAAGATTTGGCATCACACGTT)
		REV (GTTGGCCTTGGGATTGAGTGGT)
	Pv18S (CV670768.1)	FWR (GACACGGGGAGGTAGTGACAAT)
		REV (ACGAGCTTTTTAACTGCAACAACT)
	PvXDH	FWR (TTAGTTACCCATGGGGGTGTAG)
		REV (GAAGCATTAGGGACCTTGTCAG)
pET30b+ cloning	*PvXDH*	FWR (CGGGGTA<u>CCATGG</u>TATGCATTATTGCGATC)
		REV (ATAAGAAT<u>GCGGCCGC</u>TGTGTATTTGGTCCCATCTA)
	PvXDH	FWR (CGGGGTA<u>CCATGG</u>CAAACGTATTTCTGGAT)
		REV (ATAAGAAT<u>GCGGCCGC</u>AGCCATCCGAATTCTCTCA)
pFGC5941 cloning	*PvXDH*	FWR (GC<u>TCTAGAGGCGCGCC</u>AGCGTAACTTCAACTCATTTG)
		REV (CA<u>GGATCCATTTAAAT</u>ACCAACAGCTTTGGAAGAATG)

Supplementary Table S2. S-nitrosylation and Tyr-nitration sites predicted in PvXDH protein using GPS-SNO 1.0 and GPS-YNO2 1.0, respectively. Position of cysteines and tyrosines predicted to be modified are shown. Statistically significant conserved motifs in S-nitrosylation prediction were identified using the SNOsite web server.

Position	S-nitrosylation motif	Score
78	VSHYDRQLRK**C**SHYAINACLA	2,473
358	NERAAHETLS**C**KAFIEQLKWF	2,929
689	EELFAVDHVT**C**VGQVIGIVVA	3,375
1124	DMYGAAVLDA**C**EQIMTRMKPI	1,541
1343	AIIAARAEMG**C**YDWFPLDSPA	2,582

Position	Tyr-nitration motif	Score
23	DVNVSNEAIV**Y**VNGVRRVLAD	1,269
490	NWVVADASLF**Y**GGVAPYSLAA	1,229

CHAPTER 4: MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF PRAT GENES FROM *P. vulgaris*

Abstract

Purines are essential molecules generated in a highly regulated pathway in all organisms. In tropical legumes, the nitrogen fixed in the nodules is used to produce ureides through the oxidation of *de novo* synthesized purines. Glutamine phosphoribosylpyrophosphate amidotransferase (PRAT) catalyzes the first committed step of *de novo* purine synthesis. In *Phaseolus vulgaris* there are three genes coding for PRAT. The three full-length sequences, which are intron-less genes, were cloned and their expression levels were determined under conditions that affect the synthesis of purines. One of the three genes, *PvPRAT3*, is highly expressed in nodules and protein amount and enzymatic activity in these tissues correlate with nitrogen fixation. Characterization of PvPRAT1 and 2 purified after their overexpression in *Nicotiana benthamiana* reveals that, despite differences in their expression levels, PvPRAT1 and 2 are both functional enzymes, with similar kinetic parameters, and show the typical end-product inhibition by purine nucleotides. On the other hand, RNAi-silencing of *PvPRAT3* gene expression and the subsequent metabolomic analysis of the transformed roots shows that PvPRAT3 is essential for the synthesis of ureides in nodules of the so called ureidic legumes, such as *Phaseolus vulgaris*.

Introduction

Glutamine phosphoribosylpyrophosphate amidotransferase (PRAT; EC 2.4.2.14; also named ATase or GPAT) catalyzes the first reaction of the pathway for the *de novo* purine synthesis. This pathway generates IMP, AMP and GMP, that are building blocks of DNA and RNA, and also NAD(P), FAD, coenzyme A, and ATP, in all organisms (Moffatt and Ashihara, 2002; Boldt and Zrenner, 2003; Zrenner *et al.,* 2006). Apart from these essential functions, *de novo* purine synthesis also plays an important role in the primary metabolism of nitrogen in some plants, in particular in ureidic legumes. The ureides, allantoin and allantoate, which are products of purine oxidation, are the main form of nitrogen transport and storage in the so called ureidic legumes. In these legumes, atmospheric N_2, fixed as ammonium and assimilated through its incorporation to the amide group of glutamine, is incorporated to the *de novo* purine synthesis (Zrenner *et al.,* 2006). PRAT catalyzes the transfer of the ammonia group from glutamine to the activated ribose, phosphoribosylpyrophosphate (PRPP), to produce phosphoribosylamine (PRA), in the first and most highly regulated reaction of the pathway (Smith, 1998; Sugiura and Takeda, 2000) (Fig. 1). Nine subsequent enzymatic reactions are required to the synthesis of the first purine nucleotide, IMP, which then produces AMP, GMP and XMP by a few further enzymatic conversions (Zrenner *et al.,* 2006).
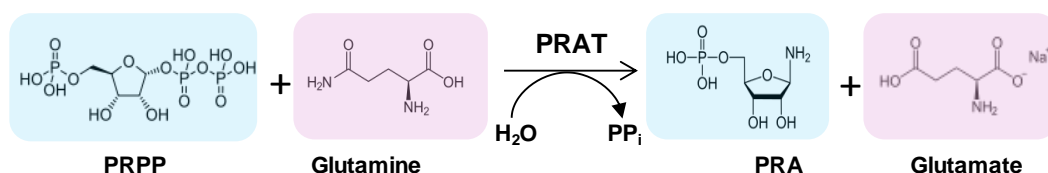
Fig. 1. Reaction catalyzed by glutamine phosphoribosylpyrophosphate amidotransferase (PRAT). The enzyme catalyzes the conversion of phosphoribosylpyrophosphate (PRPP) and glutamine into phosphoribosylamine (PRA) and glutamate.

The molecular and biochemical features of PRATs have been well studied in bacteria, yeasts and animals, but little is still known about their regulation in

plants, particularly in legumes, despite its importance in these plants. The enzyme is composed of an N-terminal glutaminase domain catalyzing the amide transfer from glutamine to PRPP and a C-terminal phosphoribosyltransferase domain. The C-terminal domain is described as the site where the binding of end products of the pathway, GMP, AMP and IMP, inhibits the enzymatic activity by competing with PRPP for binding to the enzyme. This feedback mechanism regulates the *de novo* synthesis of purines (Sugiura and Takeda, 2000). Except in *Escherichia coli* (Tso *et al.,* 1982) and yeasts (Mantsala and Zalkin, 1984b), PRATs proteins are subject to two post-translational modifications. First a small propeptide must be removed to expose an N-terminal catalytic cysteine residue, and second, an iron-sulfur [4Fe-4S] cluster is bound to four conserved cysteine residues (Souciet, 1988; Zhou, 1992). The PRATs from *Arabidopsis thaliana* have longer propeptides than such from *Bacillus subtilis* (Wong *et al.,* 1977) and animals (Rowe and Wyngaarden, 1968; Clark and MacAfee, 2000). The plant propeptides could work as signal peptides to the chloroplasts (Ito *et al.,* 1994; Kim *et al.,* 1995; Hung *et al.,* 2004), where the *de novo* purine synthesis presumably takes place (Atkins *et al.,* 1997; Zrenner *et al.,* 2006).

As a result of its prominent role in ureidic legumes, the first PRAT protein characterized from a plant source was that from soybean nodules (Reynolds *et al.,* 1984). The characteristic N-terminal propeptide and the [4Fe-4S] cluster were identified in the polypeptide sequences deduced from cDNAs expressed in soybean and moth bean functional nodules, and the two enzymes showed inhibition by ends products of the pathway, indicating common regulation features among all PRAT proteins (Reynolds *et al.,* 1984; Kim *et al.,* 1995). On the other hand, although the enzymes required for the synthesis of purines are found in tissues other than nodules, the *de novo* purine pathway was highly induced in nodules of ureide-transporting legumes (Atkins, 1991; Atkins and Smith, 2000). Although the exact mechanism of induction remains unclear, the activity of several enzymes of the biosynthetic pathway is related with the flux of fixed nitrogen and treatments that inhibited N_2 fixation exerted a negative effect

(Smith *et al.,* 2002). Moreover, treatment with exogenous glutamine led to the induction of *PRAT* expression in soybean roots, further supporting the importance of this enzyme in the regulation of the *de novo* synthesis of purine nucleotides from the nitrogen fixed in nodules (Kim *et al.,* 1995).

Besides the reports on nodule specific PRATs of the ureidic legumes soybean and mothbean, the characterization of PRATs from plants is limited to the *ATase* gene family from *A. thaliana*. In Arabidopsis, the enzyme is encoded by three genes. *ATase1* and *ATase2*, encode polypeptides that, despite a high degree of identity, showed different tissue-specific expression patterns. *ATase1* was expressed in roots, flowers and slightly in leaves, whereas *ATase2* was the predominant form expressed in leaves (Ito *et al.,* 1994). The sequencing of Arabidopsis genome revealed a third *ATase* gene. *ATase3* was expressed at very low levels in siliques, cauline leaves and roots (Boldt and Zrenner, 2003). Mutational studies in *A. thaliana* showed the relevant role of *ATase2* in growth, cell division and chloroplast biogenesis (Hung *et al.,* 2004). *ATase2* is the main functional isoform in most tissues, as revealed by the small and albino/pale-green mosaic leaves of *ATase2* mutants, whereas phenotype of the other two mutants was inconspicuous, although *ATase1* overexpression restored the wild-type phenotype in *ATase2* mutants (Hung *et al.,* 2004).

In this work, molecular and biochemical tools have been generated and used for functional characterization of *P. vulgaris* PRATs. The three PRAT coding sequences found in common bean genome have been isolated, the coded polypeptides have been characterized, and the expression patterns under conditions affecting purine synthesis have been analyzed. Moreover, the PvPRAT proteins have been over-expressed in *Nicotiana benthamiana*, and the properties of the purified enzymes have been determined. *PvPRAT3* was the predominant isoform expressed in nodules from *P. vulgaris*. To study the function of PvPRAT3, the gene was silenced by RNAi using a *P. vulgaris* hairy root transformation system. Metabolomic and transcript analysis of silenced roots demonstrated that PvPRAT3 plays a key role for the utilization of symbiotically

fixed nitrogen in *P. vulgaris.* Specifically, PvPRAT3 isoform is thought to provide the first committed step for ureide biosynthesis in the nodules of ureidic legumes.

Materials and methods

1. Plant material and growth conditions

Phaseolus vulgaris L. cv Great Northern seeds were surface-sterilized and germinated in Petri dishes under sterile conditions. After germination, seedlings were sown on pots filled with vermiculite/perlite mixture (2/1 w/w) and inoculated with a fresh suspension of *Rhizobium leguminosarum* ISP14 which had been cultured at low temperatures (28 °C) for less than 30 hours.

Lotus japonicus cv Gifu and *M. truncatula* cv Jemalong A17 seeds were scarified, surface-sterilized and germinated in Petri dishes on agar-solidified medium. Plantlets were transferred to pots filled with vermiculite and inoculated with a fresh suspension of *Mesorhizobium loti* JA76 for *Lotus* and *Sinorhizobium meliloti* 2011 for *Medicago* plants.

Plants were cultured in growth chamber under a long-day photoperiod (16 hours light, 8 hours dark), 300 $\mu E.m^{-2}.s^{-1}$ lighting, and 70% relative humidity at 26-21°C (day-night temperatures). Inoculated plants were watered three times a week with nitrogen-free nutrient solution (Rigaud and Puppo, 1975).

Nicotiana benthamiana plants were sown on vegetal substrate and cultured in a growth chamber under the same conditions described above. In the experiments to study the effect of nitrate on the expression of *PvPRAT*s, *P. vulgaris* plants were randomly distributed into three groups at sowing. One group was watered with nutrient solution containing 10 mM KNO_3 for 28 days and the rest were inoculated with rhizobia and watered with the nitrogen-free nutrient solution for 24 days. Afterwards, nutrient solution containing 10 mM KNO_3 was used to irrigate half of the N_2-fixing plants until 28 days after sowing, whereas the other half was kept at the nitrogen-free watering regime until the end of the experiment. To study the effect of development on the regulation of PvPRAT, plants of *P.*

vulgaris were cultured under N_2-fixation or in KNO_3 fertilization conditions and whole plant tissues were collected at 21, 28, 35, 42 and 49 days after sowing. In some experiments, the primary leaves (oldest) and the uppermost (youngest), leaves were also collected, and stored as independent samples. Nodules collected from each individual plant were weighed and photographed, as a visual control of N_2 fixation capacity among the different plants and experiments. Samples were collected from 4-6 plants per treatment/sampling and each experiment was repeated at least 3 times. Plant material was collected at indicated times, frozen with liquid nitrogen and stored at -80 ℃ till further analysis.

2. Nitrogen fixation measurement

Nitrogenase (EC 1.7.9.92) activity was measured as the representative H_2 evolution in an open-flow system (Witty and Minchin, 1998) using an electrochemical H_2 sensor (Qubit System Inc., Canada). For that, nodulated roots excised from their shoots just before the activity assay were sealed in 0.125 L cylinders and H_2 production was recorded according to the manufacturer´s instructions. Apparent nitrogenase activity (ANA, rate of H_2 generation in air) was determined under N_2:O_2 (80%:20%) with a total flow of 0.4 L.min^{-1}. After reaching steady-state conditions, total nitrogenase activity (TNA) was determined under Ar:O_2 (79%:21%). Standards of high-purity H_2 were used to calibrate the detector. Roots attached to whole plants were also measured to ascertain that the separation from shoots did not cause a significant reduction on nodule activity during the assay. Data are given as nitrogen fixed per plant.

3. Isolation of *PvPRAT* coding sequences

Three possible PRAT coding sequences in common bean genome could be identified in the databases, although the information concerning full-length transcripts was at that time fairly incomplete. A sequence of 807 bp from the start codon of *PvPRAT3* gene was isolated with primers designed according to ESTs sequences from *P. vulgaris* expressed in nodules. Total RNA obtained from leaves

and nodules was used to isolate the 5´-, and 3´-ends of the *PvPRAT1 and PvPRAT3* genes using gene specific primers (Supplementary Table S1) and the *SMART^{TM}RACE* kit (*Rapid Amplification of cDNA-ends*, Clontech). For the isolation of the full-length coding sequence of *PvPRAT2*, primers were designed according to *Phytozome* database information (http://phytozome.jgi.doe.gov/pz/portal.html). *SMART^{TM}RACE* was then used to determine the 5´UTR and 3´UTR sequences of *PvPRAT2* using gene specific primers (Supplementary Table S1). The resulting PCR products of partial and full-length cDNAs were cloned into pGEM-T easy vector (Promega, Madison WI) and their identity verified by sequencing several independent clones from each construct.

4. Gene expression analysis

Total RNA was isolated from the different tissues using the TRI REAGENT (Sigma-Aldrich) following the manufacturer's instructions. Prior to RT-PCR, total RNA from control and treated tissues was treated with RNAase-free DNAaseI (Promega, Madison WI) at 37 ℃ for 30 min to eliminate any traces of genomic DNA. Lack of 18S rRNA amplification was used to check the successful removal of DNA. First strand cDNA synthesis was done with 2.5 µg of DNAase-treated RNA using iScriptP^{TM} reverse transcriptase (Bio-Rad) following the manufacturer's instructions. Expression analysis was performed by quantitative RT-PCR in an iCycleriQ System (Bio-Rad, Hercules, CA, USA) using the iQ SYBR-Green Supermix (Bio-Rad) gene specific primers (Supplementary Table S1). The PCR programme consisted of an initial denaturation and Taq polymerase activation step of 5 min at 95 ℃, followed by 50 cycles of 15 s at 95 ℃ and 1 min at 60 ℃. Expression of *Actin-2* and 18S genes was used as internal controls, after checking that its level of expression was similar in all samples. Specificity of primers was confirmed by dissociation curves analysis and by sequencing of the PCR products. The amplification efficiency of each primer pairs, calculated by PCR using serial dilutions of root and leaf cDNAs, was >

98%. Analysis of relative gene expression was calculated from ΔCT values (Livak and Schmittgen, 2001), using expression of *Actin-2* gene for data normalization. All the reactions were set up in triplicate (three technical replicates) using three to five RNA preparations from tissues collected from independent experiments (biological replicates).

5. PRAT activity assay

For the enzymatic assay of PRAT activity, a slightly modification of the method described by Walsh *et al*. (2007) was used. This method is based in the continuous spectrophotometric determination of the NADH produced in a reaction mix in which glutamate dehydrogenase (GDH) activity was coupled to the PRAT-released glutamate. All procedures for protein extracts preparation and enzyme manipulations were carried out at 0–4 °C. Frozen plant material was ground to a fine powder under liquid nitrogen. Plant extracts were obtained by adding 3 mL of extraction buffer per gram of tissue. The extraction buffer for PRAT assay was 100 mM Tea-NaOH, pH 8.0; 150 mM NaCl; 15 mM DTT; 1 mM $MgCl_2$ and 0.5% (w/v) Triton X-100. The resulting homogenate was centrifuged at 15000 *g* for 10 min and the supernatant was dialyzed through *SpinTrap G25* columns previously equilibrated with 100 mM Tea-NaOH, pH 8.0; 5 mM DTT and 1 mM $MgCl_2$ buffer, and the flow-through was used as crude extract.

The enzymatic assay was carried out at 30 ºC, in a reaction mixture composed of 100 mM Tea-NaOH, pH 8.0; 5 mM DTT; 1 mM $MgCl_2$; 1 mM PRPP; 8 mM glutamine; 6 mM NAD^+; 1U GDH and 5% (v/v) of the previously dialyzed crude extract. PRAT activity was determined following the continuous production of NADH by GDH-coupled assay during 5 min. In parallel, all the measurements were carried out without the substrate PRPP, as a control of unspecific glutaminase activity. Soluble protein was measured in the same extracts in which the enzymatic activity was determined (Bradford, 1976).

The results are expressed as means of the values from at least three independent

experiments. Enzymatic assays and analytical determinations from each biological experiment were done at least twice (two independent samples) and each assay was measured by duplicate (technical replicate).

6. Ureide determination

The concentration of ureides was determined by the colorimetric assay of glyoxylate derivatives (Vogels and Van der Drift, 1970) in which allantoin and allantoate are determined after their chemical transformation to glyoxylate.

For each assay, 100 mg of plant tissue were homogenized in 0.4 mL of 50 mM Tris–HCl, pH 7.8; 100 mM $MgSO_4$ and 0.15% (w/v) deoxycholic acid (DOC) and centrifuged at 15000 g for 10 min. The supernatant was immediately used for ureide and protein determination (Bradford, 1975). For each biological experiment, determinations were done by duplicate (technical replicate).

7. Transient expression in leaves of *Nicotiana benthamiana* and purification of the PvPRATHA.Strep-tagged proteins

For their transient expression in *N. benthamiana*, the whole coding sequences of *PvPRAT1*, *PvPRAT2* and *PvPRAT3*, including their targeting signal peptides, were amplified by PCR using the primers pairs for each gene listed in Supplementary Table S1. For the expression of the cytosolic version of *PvPRAT3*, primers were used to obtain a polypeptide product starting at position 64, 11 amino acids before the catalytic Cys-1 residue. Each of these primer pairs introduce a *Cla*I and *Sma*I restriction sites, respectively, used for cloning the PCR products into the binary vector pXCS.HA.StrepII (AY457636; Witte *et al.,* 2004), and the resulting plasmids were introduced into *Agrobacterium tumefaciens* GV3101::pMP90RK (Koncz and Schell, 1986) and used to agro-infiltrate *N. benthamiana* leaves. Four days after *Agrobacterium* infiltration, leaf discs were harvested, frozen in liquid nitrogen and stored at -80 °C until used. PvPRAT1-3.HA.Strep proteins were purified according to (Werner *et al.,* 2008) with some slight modifications. About 0.75 g of *N. benthamiana* leaves were ground in 1.5

mL of extraction buffer (100 mM Tea-NaOH, pH 8.0; 150 mM NaCl; 15 mM DTT; 1 mM $MgCl_2$ and 0.5% (v/v) Triton X-100) and the extract was centrifuged at 15000 g for 10 min at 4 ℃. The supernatant was collected and 40 µL of Strep-tactin sepharose suspension (GE Healthcare) were added. Binding was performed by incubation of approximately 1.5 mL of this mixture for 10 min at 4 ℃ with gentle shaking in 1.5 mL eppendorf tube. The mixture was collected by centrifugation at 700 g for 30 s at 4 ℃ and the supernatant was discarded. The resin was washed five times with 0.5 mL of wash buffer (100 mM Tea-NaOH, pH 8.0; 150 mM NaCl; 5 mM DTT; 1 mM $MgCl_2$ and 0.005% (v/v) Triton X-100). PvPRAT1-3 proteins were eluted with 0.1 mL of elution buffer (100 mM Tea, pH 8.0; 150 mM NaCl; 5 mM DTT; 1 mM $MgCl_2$; 0.005% (v/v) Triton X-100 and 2.5 mM desthiobiotin) after incubation for 5 min at low temperature with gentle shaking.

8. Western blot analysis

Proteins were separated by 10% SDS-PAGE (Laemmli, 1970) in a Mini PROTEAN III system (BioRad). After separation, the proteins were electro-transferred to polyvinylidene fluoride membrane (Sigma). To detect PvPRAT proteins, blots were incubated with polyclonal antibodies raised against recombinant PvPRAT1 produced in bacteria, at a 1:1000 dilution, or with a monoclonal antibody against hemagglutinin (HA) epitope at a 1:5000 dilution. Anti-rabbit or mouse IG, alkaline phosphatase-conjugated (Sigma), were used as secondary antibodies at a 1:12000 dilution and the reaction was developed using 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (BCIP) and nitro-blue tetrazolium chloride (NBT). The specificity of the antibodies against PRAT protein was tested by labelling blots with preimmune serum.

9. Silencing of *PvPRAT3* gene transcription and plant transformation

To generate the silencing hairpin RNA construct, a fragment from the 3´ region of *PvPRAT3* cDNA was cloned into pFGC5941 vector (AY310901; Kerschen *et al.,*

2004). For that, 388 base pairs of cDNA were amplified by PCR using a forward primer containing *Xba*I and *Asc*I restriction sites and a reverse primer containing *BamH*I and *Swa*I restriction sites (Supplementary Table S1). The resulting PCR products were cloned using *Asc*I and *Swa*I sites to insert the fragment in sense orientation into pFGC5941, upstream of an intron, and the *Bam*HI and *Xba*I sites for cloning the antisense fragment, downstream of the intron. The resulting construct was verified by sequencing and introduced into the strain K599 of *Agrobacterium rhizogenes*.

Common bean plantlets were transformed according to (Estrada-Navarrete *et al.,* 2007). Once hairy roots appeared, the primary roots were excised by cutting approximately 1 cm below the cotyledonary nodes, and plants were sown and inoculated with *Rhizobium tropici* strain j, by adding 1 mL of the culture directly to the transgenic roots. Plants were watered with B&D nitrogen-free solution and about four weeks later roots and nodules were harvested and frozen in liquid nitrogen.

10. Plant metabolite profiling by gas chromatography-electron impact-time of flight mass spectrometry (GC-MS)

Control transformed roots and *PvPRAT3* silenced roots were harvested, and 100 mg fresh weight (+/- 5%) were frozen and homogenized in liquid nitrogen. Six replicates were prepared for each condition. Samples were prepared and analyzed essentially as described by Erban *et al.* (2007) and Allwood *et al.* (2009). Specifically, 360 mL of a precooled mixture, i.e., 300 mL of gas chromatography grade methanol, 30 mL of 2 mg.mL^{-1} nonadecanoic acid methyl ester in chloroform, 30 mL of 1mg.mL^{-1} [$^{13}C_6$]-sorbitol in distilled water, was added to the samples. After 15 min of shaking at 70 ºC, 200 µL of chloroform was added and the samples were incubated at 37 ºC for 5 min. Then, 400 µL of distilled water was added, the solvents mixed, and phases separated by centrifugation at 15000 *g* for 5 min. Samples of 160 µL from the upper polar phase were carefully withdrawn, transferred to new 1.5 mL micro-centrifuge tubes and dried using

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speed vacuum without heating.

Dried samples were stored at -20 ℃ until use. Metabolite analysis was done after the chemical derivatization of the samples by methoxyamination and subsequent trimethylsilylation. Samples were incubated for 1.5 hours at 37 ℃ with 40 µL of a solution containing 40 mg methoxyaminehydrochloride per 1 mL pyridine. Afterwards, 80 µL of BSTFA-premixture, consisting of 7/1 (v/v) N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) and alkane-mixture, was added (Erban *et al.,* 2007). Measurements were done with an Agilent 6890N24 gas chromatograph (Agilent Technologies, Böblingen, Germany; http://www.agilent.com) using a FactorFour VF-5ms capillary column of 30 m length, 0.25 mm inner diameter, 0.25 µm film thickness (Varian- Agilent Technologies) with a 10 m Integra guard-column. The gas chromatograph was connected to a Pegasus III time-of-flight mass spectrometer (LECO Instrumente GmbH, Mönchengladbach, Germany; http://www.leco.de). All analyses were conducted in splitless mode with 1 µL injection volume and helium as carrier gas, at a flow rate of 0.6 mL.min^{-1}. Injector temperature was set to 230 °C. The initial temperature of the GC-program was 70 ℃ kept for 1 min. Then temperature was ramped to 350 ℃ at a rate of 9 ℃.min^{-1} and held at 350 ℃ for 5 min.

11. Metabolite identification and quantification

GC-MS data processing into a standardized numerical data matrix and compound identification were performed using the TagFinder software (Lüdemann *et al.,* 2008; Strehmel *et al.,* 2008). Compounds were identified by mass spectral and retention index matching to the reference collection of the Golm metabolome database (GMD; Kopka *et al.,* 2005; Hummel *et al.,* 2010). Each mass feature was normalized to sample fresh weight and internal standard sorbitol. Hierarchical clustering was performed in data of *PvPRAT3* silencing roots calculated relative to data of control transformed roots using Multiexperiment Viewer version 3.1 with a matrix based on Pearson correlation.

12. Statistical analysis of the data

Student´s t-test was performed using the GraphPad software package.

RESULTS

1. PRAT coding sequences from *Phaseolus vulgaris*

Southern-blot analysis, performed as a preliminary part of this work, revealed the presence of three PRAT coding genes in *P. vulgaris* (Supplementary Fig. S1). However, only the sequences of the *PvPRAT1* and *PvPRAT3* genes were originally identified by rapid amplification of cDNA ends (RACE) using leaf and nodule specific ESTs, respectively. The presence of three sequences apparently coding for PRAT proteins was confirmed by the recently released genome sequence from *P. vulgaris* (Schmutz *et al.,* 2014). Full-length *PvPRAT2* cDNA sequence was then isolated using *Phytozome* sequences information. Once the *PvPRAT* cDNAs were obtained, 5´ RACE was also done to determine transcription origins and proximal 5' regulatory sequences that were not defined in the database (Supplementary Fig. S3). Interestingly, comparison of cDNA and genomic sequences revealed that none of the three putative PRAT coding genes from *P. vulgaris* contained introns within their sequences (Supplementary Table S2).

The deduced peptide sequences of the three PvPRAT proteins showed the N-terminal glutaminase and the C-terminal phosphoribosyltransferase differentiated domains involved in the characteristic two-step reaction of glutamine amidotransferases. The three PvPRAT coding sequences exhibited a high degree of identity, particularly *PvPRAT1* and *2,* with a 96.3% similarity. *PvPRAT3* was the most divergent sequence with 75.7 and 76.1% similarity to *PvPRAT1* and *2,* respectively. The deduced ORFs from the *PvPRAT1*, 2 and *3* cDNAs were 1677, 1662 and 1728 bp, coding for 558, 553 and 575 amino acids peptides, with an estimated molecular mass of 60.2, 59.7 and 62.9 kDa, respectively (Fig. 2).
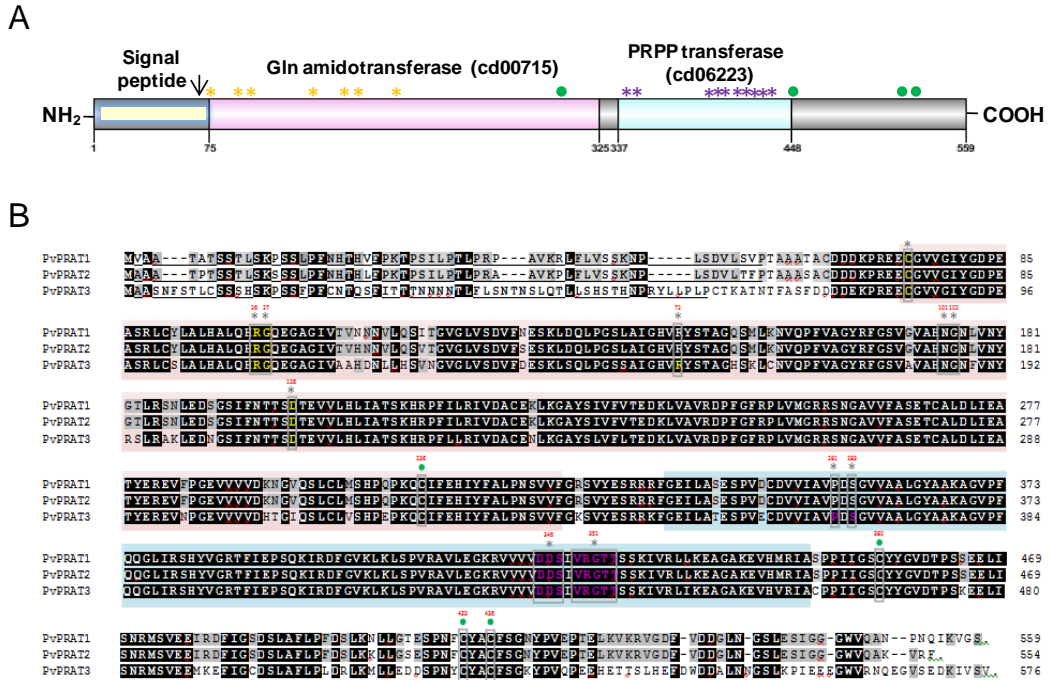
Fig. 2. Organization of PRAT proteins from *P. vulgaris*. (A) Schematic representation. Positions of the two characteristic domains (PFAM: cd00715 and cd06223) are indicated. (B) Aligment of the deduced amino acid sequences of the three PRAT proteins identified in *P. vulgaris*. Predicted glutamine amidotransferase and phosphoribosyltransferase domains are indicated by purple and blue shaded boxes, respectively. Shaded in black are shown the residues identical in the three proteins and residues conserved in two protein sequences appear grey-shaded. Predicted targeting signal peptide is underlined. Boxes and asterisks denote invariable residues on active sites. The four 4Fe-4S ligand cysteine residues are denoted by boxes and green dots.

A long putative signal peptide was identified in the N-terminal sequence of the PvPRATs, with a high prediction score to chloroplast import, and a length of 60 residues for PvPRAT1 and 2 and of 64 residues for PvPRAT3.

The three *P. vulgaris* PRAT proteins contained the conserved cysteine residue, at position 75 in PvPRAT1 and 2, and at position 86 in PvPRAT3, that should become the N-terminal cysteine residue after the autocatalytic removal of an eleven amino acids propeptide in the homologous protein from *B. subtilis,* as well as in many other microbial and eukaryotic PRATs. Moreover, the PvPRATs amino acid sequences also contain the four conserved cysteine residues at positions 310,

456, 507 and 510 in PvPRAT1 and 2, and positions 321, 467, 518 and 521 in PvPRAT3 (Fig. 2), that were described to bind the [4Fe-S] cofactor in the *B. subtilis* GPRAT (Zalkin and Dixon, 1992), and that are also found in the PRAT proteins from soybean nodules (Kim *et al.,* 1995).

2. Differential expression of *PRAT* genes in *P. vulgaris* tissues

To study the relative contribution of each *PRAT* gene to the synthesis of purine nucleotides in *P. vulgaris,* qRT-PCR and gene specific primers (Supplementary Table S1) were used to measure the expression level of the three *PvPRAT* genes under conditions that may affect the synthesis *de novo* of purines. For that, cDNAs were prepared from tissue samples collected at 28 days after sowing, time in which the synthesis of ureides is fully activated in plants cultivated under N_2-fixation conditions. As is shown in Fig. 3A, the expression of *PvPRAT3* in nodules highly exceeded that of *PvPRAT1* and *2*. In contrast, *PvPRAT1* was preferentially expressed in the trifoliate leaves (Fig. 3B). On the other hand, *PvPRAT2* exhibited a low level of expression in all the tissues analyzed so far. Apart of its extraordinary high expression in nodules, *PvPRAT3* was expressed in all plant tissues, resembling the pattern of *PvPRAT1,* except in roots, where *PvPRAT3* gene showed higher expression level than any of the other two *PvPRAT* genes (Fig. 3B).

Besides the transcript expression, the actual protein and enzymatic activity levels were determined in *P. vulgaris* tissues. A protein band at the expected size for PRAT (53-55 kDa) was detected by the polyclonal antibodies raised against the full-length PvPRAT1 protein (Fig. 3C). The intensity of the band was higher in nodules than in leaves, which indicates that the antibodies were able to recognize PvPRAT3 isoform. Surprisingly, there was also an intense band in stems, where the expression level of the three PRAT coding transcripts was lower than in nodules and leaves. Moreover, an intense band of higher molecular mass was immunodetected in roots, where only an extremely faint band of the 53-55 kDa for the mature PRAT proteins was found (Fig. 3C). The PRAT activity was

measured in crude extracts using a modification of the assay described by Walsh *et al.* (2007). In this enzymatic assay, based on glutamate production, unspecific glutaminase activity may mask the actual PRAT activity determination. Therefore, control glutaminase assays, in which glutamate produced in absence of PRPP is measured, were carried out along-side with the PRAT assays. As shown in Fig. 3D, the highest level of PRAT activity was found in nodules, followed by activity in leaves. In contrast, specific PRAT activity was low in roots and stems, although these tissues presented high glutaminase activities.
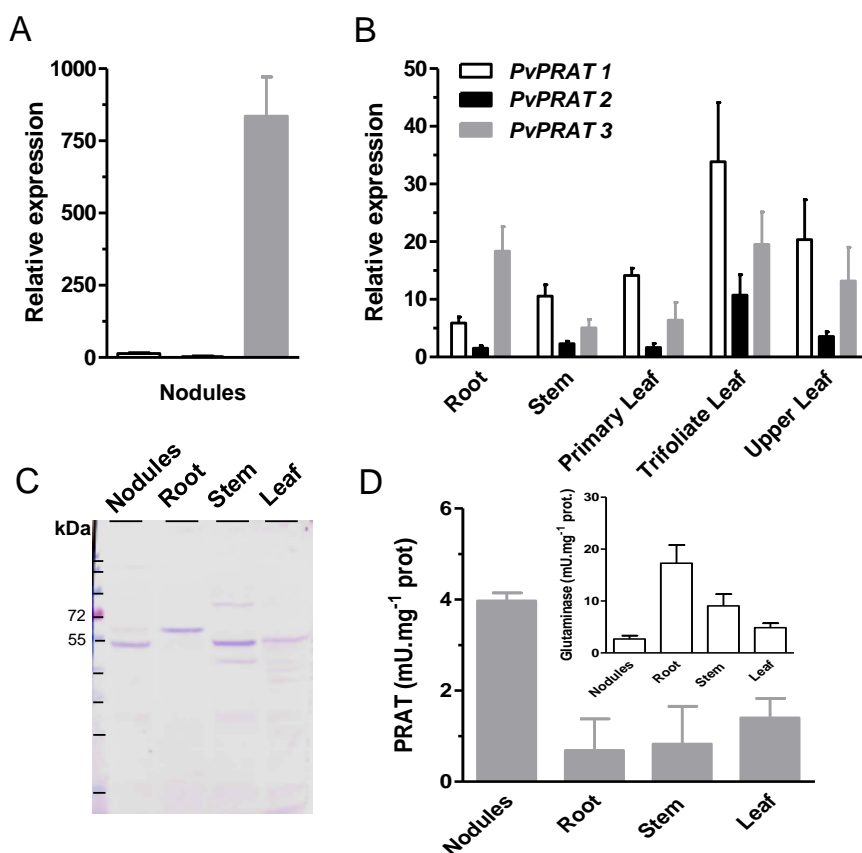
Fig. 3. PRAT expression, protein and activity in tissues from *P. vulgaris*. (A) Relative transcript expression of *PvPRAT1*, *2* and *3* in nodules. (B) Relative transcript expression of *PvPRAT1*, *2* and *3* in root, stem, and leaves. Data were normalized to the expression of the *Actin-2* gene. (C) Western blot analysis of PvPRAT proteins in *P. vulgaris* tissues, probed with anti-PvPRAT1 polyclonal antibodies. (D) Specific PRPP-dependent PRAT activity and unspecific glutaminase activity (inside), in nodule, root, stem and trifoliate leaf. Tissue samples were collected at 28 days after sowing from plants grown under conditions of N_2-fixation.

3. Expression of *PvPRAT3* is related to N$_2$ fixation activity

To investigate the relationship of *PvPRAT* genes and N$_2$-fixation, their expression patterns were compared among plants that were grown under conditions of N$_2$ fixation and plants grown under fertilization with nitrate (Fig. 4). In the leaves, expression patterns of *PvPRAT1* and *2* were not significantly affected by the nitrogen condition of the plants (Fig. 4A and 4B). In contrast, there was a significant reduction in *PvPRAT3* expression level under nitrate fertilization in comparison to the expression under N$_2$-fixing conditions (Fig 4C). *PvPRAT1* was the gene most highly expressed in leaves, showing an expression peak at 28 days after sowing (Fig 4A), whereas expression of *PvPRAT2* remained at a constant low level, independently of the culture condition (Fig. 4B). In the leaves from N$_2$-fixing plants, *PvPRAT3* transcript level increased gradually until 42 days after sowing, reaching levels close to those of *PvPRAT1*, and decreased at 49 days, (Fig. 4C). However, the leaves from plants fertilized with nitrate showed low levels of *PvPRAT3* expression along the whole period analyzed, resembling the basal expression found for *PvPRAT2* under either growth conditions (Fig.4B and 4C).

Furthermore, expression of *PvPRAT* genes was measured at 21, 28, 35, 42 and 49 days after sowing in the nodules from N$_2$-fixing *P. vulgaris* plants. As shown in Fig. 4D and 4E, expression of *PvPRAT1* and *2* followed similar patterns, although *PvPRAT2* was expressed at a level about twenty times lower than *PvPRAT1*. As expected from previous results, *PvPRAT3* exhibited a much higher expression in nodules than *PvPRAT1* and *2* (Fig. 4F). Moreover, the level of enzymatic PRAT activity measured in the nodules, shown in Fig 4G, agreed with the expression of *PvPRAT3* in these tissues, and was similar to the temporal pattern of nodule dry mass (Fig 4H) and apparent nitrogen fixation (ANA) (Fig 4I). Surprisingly, the expression levels were lower in nodules of 35 days than in those of 28 and 42 days for all three PRAT coding genes, although nodule mass and N$_2$ fixation activities were at their highest levels at 35 days (Fig. 4H and 4I). Nevertheless, the specific PRAT activity increased from 21 to 35 days, time in which nodule

BNF activity begins to decline (Fig. 4G).
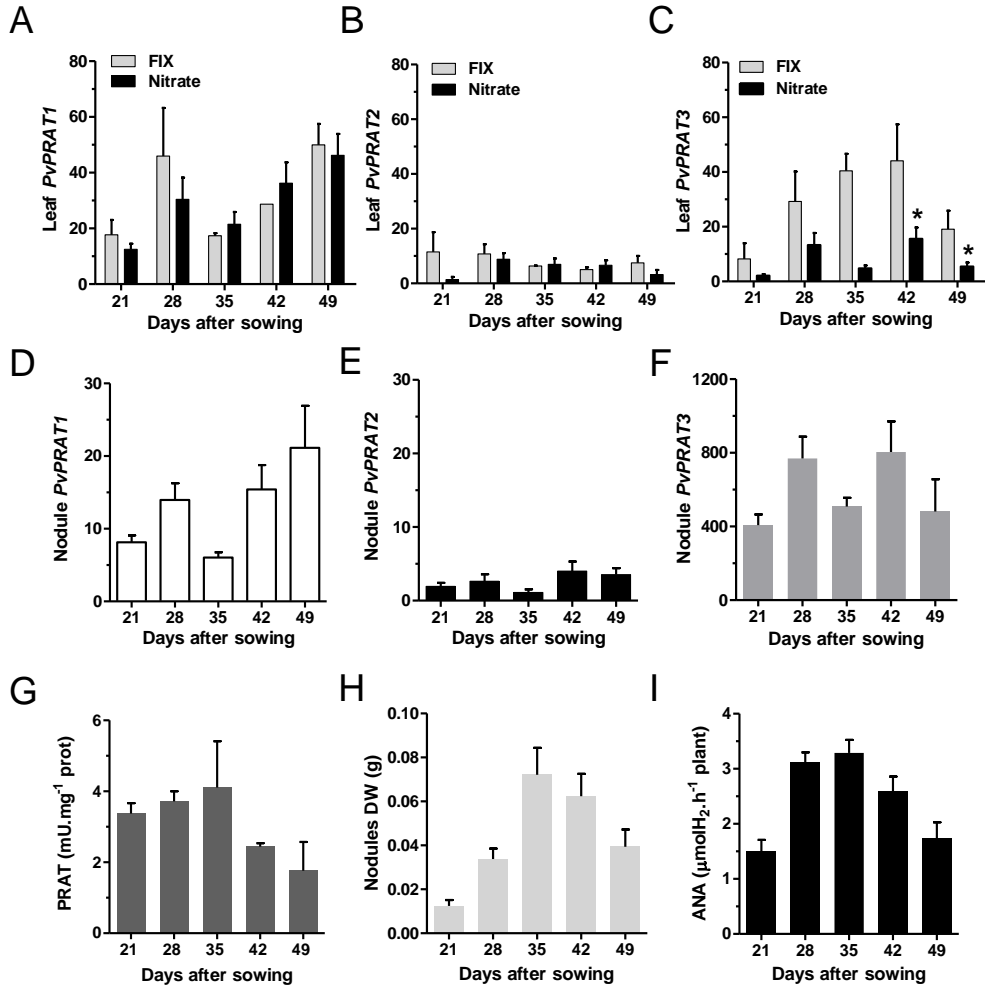
Fig. 4. N₂-fixation effects on the expression patterns and activity of PvPRATs. (A-C) Relative transcript expression of *PvPRAT1*, *2* and *3* in leaves from plants grown under N₂ fixation conditions (grey bars) or plants nitrate-fertilized (black bars). (D-F) Relative transcript expression of *PvPRAT1*, *2* and *3* in nodules. Data were normalized to the expression of the *Actin-2* gene. (G) Specific PvPRAT activity in nodules. (H) Nodule dry weight (DW). (I) BNF measured as apparent nitrogenase activity (ANA). Samples were collected at 21, 28, 35, 42 and 49 days after sowing. Significant differences according to Student´s *t*-test are denoted by asterisks (* $p<0.05$).

4. Short-time nitrate fed reduce PvPRAT activity

Symbiotic N₂-fixation and ureide production from *de novo* purine synthesis are inhibited after the feeding of N₂-fixing *P. vulgaris* plants with inorganic nitrogen (Díaz-Leal *et al.,* 2012). To further ascertain whether PvPRAT3 function is linked

to N_2 fixation, three weeks-old plants cultured under N_2-fixing conditions were irrigated with 10 mM nitrate during 4 consecutive days, and *PRAT* expression and activity were determined in the treated samples (Fig. 5).
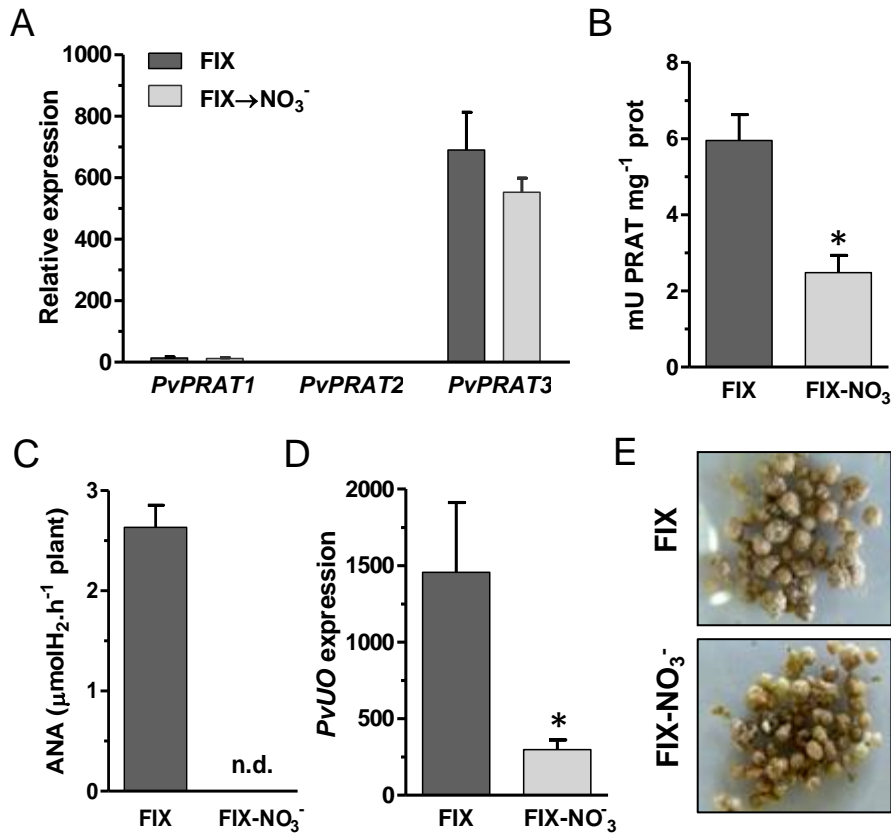
Fig. 5. Effect of short-term nitrate treatment in PRAT level, N_2 fixation and uricase (*UO*) expression in nodules from *P. vulgaris*. (A) Relative transcript expression of *PvPRAT1*, *2* and *3*. Data were normalized to the expression of the *Actin-2* gene. (B) PRAT activity. (C) Apparent nitrogenase activity (ANA). (D) Relative transcript expression of uricase (UO). (E) Control and nitrate-treated nodules. Plants cultivated under N_2-fixation conditions until 26 days after sowing were irrigated during three consecutive days with 10 mM KNO_3 (FIX-NO_3^-) or kept under the N_2-fixation (FIX). Nodules samples were analyzed at 28 days after sowing. Significant differences according to Student´s *t*-test are denoted by asterisks (* $p<0.05$).

Upon short-time nitrate treatment there was not a significant reduction in the expression levels of any of the three *PRAT* genes (Fig. 5A). However, enzymatic PRAT activity was reduced to about 60% (Fig. 5B) and, as expected, N_2 fixation decreased to undetectable levels upon nitrate treatment (Fig. 5C). As a control of

the negative effect of nitrate in ureide synthesis, expression level of *PvUO* gene, encoding uricase, was analyzed in these nodules. The level of *PvUO* transcripts diminished about 80% in plants irrigated with nitrate (Fig. 5D). Moreover, despite the short-time treatment used, there were already symptoms of senescence, as reduction in size and darkening, in the nitrate-treated nodules (Fig. 5E).

5.   Overexpression and purification of recombinant PvPRAT proteins

To ascertain that the three isolated cDNA sequences encoded functional PRAT enzymes, the coding regions of *PvPRAT1*, *2* and *3* genes were cloned into the plant expression vector pXCS.HA.StrepII and transiently expressed as C-terminal HA.Strep-fusion proteins in *Nicotiana benthamiana* (Witte *et al.,* 2004). Leaves from *N. benthamiana* were infiltrated with a mixture of *Agrobacterium* cultures carrying the corresponding pXCS.HA.Strep-PvPRATs construct and the pBIN61-P19 vector, which encode the silencing suppressor protein P19, to boost protein expression. As is shown in Fig. 6A, in the crude extracts from agro-infiltrated leaves, antibodies against the HA-epitope detected proteins of about 55 kDa for the PvPRAT1 and 2 expression constructs, corresponding to the expected size of the mature PRATs without the plastid import sequences and with a C-terminal HA-Strep fusion, although there was a much larger amount of protein in the PvPRAT1 expressing extracts than in the PvPRAT2 ones. However, only a larger molecular mass, of about 66 kDa protein was detected with the anti-HA antibody in the PRAT3.Ha.Strep samples (Fig. 6A).

One of the differences among PvPRAT proteins is the longer signal peptide of the PvPRAT3. To test whether this could affect the expression in the leaves of *N. benthamiana,* a construct was obtained to express the PvPRAT3 protein in the absence of the putative signal peptide. Removal of the signal peptide sequence led to a higher expression of PvPRAT3 (CytPvPRAT3.HA), than the previous construct (Fig. 6A, last lane). However, the protein appeared also as the ≈66 kDa form, thus indicating that the signal peptide had been already processed from PvPRAT3.HA, and that the larger molecular mass protein was probably generated

by posttranslational modification of the PvPRAT3 protein when expressed in leaves (Fig. 6A).

Moreover, besides the protein band of 55 kDa, corresponding to the expected size of the mature PRATs, the HA antibodies recognized two additional bands in the samples expressing PvPRAT1 and 2 proteins, a ≈60 kDa band that could correspond to unprocessed forms, still carrying the long signal peptide of about 6-8 kDa, and a faint band, 10-12 kDa larger than the expected size of the mature recombinant PRATs, which coincided in size with the major proteins expressed from the PvPRAT3 constructs (Fig. 6A).

The HA.Strep-tagged PvPRATs were purified by affinity chromatography, following reported procedures (Witte *et al.,* 2004). This method allowed the fast and highly reproducible purification, to electrophoretic homogeneity, of the PvPRATs recombinant proteins. The two larger proteins, of about 60 ± 1.5 kDa and 66 ± 2.5, were also found, although purification largely enriched the mature, 55 kDa forms the PvPRAT1 and 2 samples. In contrast, the 66 kDa protein bands were the most abundant in the PvPRAT3 samples, while only a faint band corresponding to the molecular mass of the mature protein was detected (Fig. 6B). As expected, the purified polypeptides were also detected when polyclonal antibodies raised against PvPRAT1 were used. Anti-PvPRAT1 antibodies led also to the detection of faint bands corresponding to the largest molecular mass proteins (Fig. 6C). Specificity of expressed proteins was assessed by the absence of HA-immunoreactive polypeptides in control, non-inoculated *N. benthamiana* leaves, or leaves infiltrated with *Agrobacterium* carrying an empty pXCS.HA.StrepII vector.

To confirm that the three isolated cDNAs encoded functional PRATs, the enzymatic activity of each PvPRAT.HA.Strep protein was determined using crude extracts from *N. benthamiana* leaves expressing the recombinant proteins, or the affinity purified PvPRATs. Specific, PRPP-dependent, PRAT activity was determined in samples from leaves infiltrated with the PvPRAT1 and 2 expressing constructs, but remained at the background level in the PvPRAT3 infiltrated

samples (Fig. 6D and 6E), probably due to the low amount of the mature protein found for the PvPRAT3 constructs (Fig. 6A-6C).
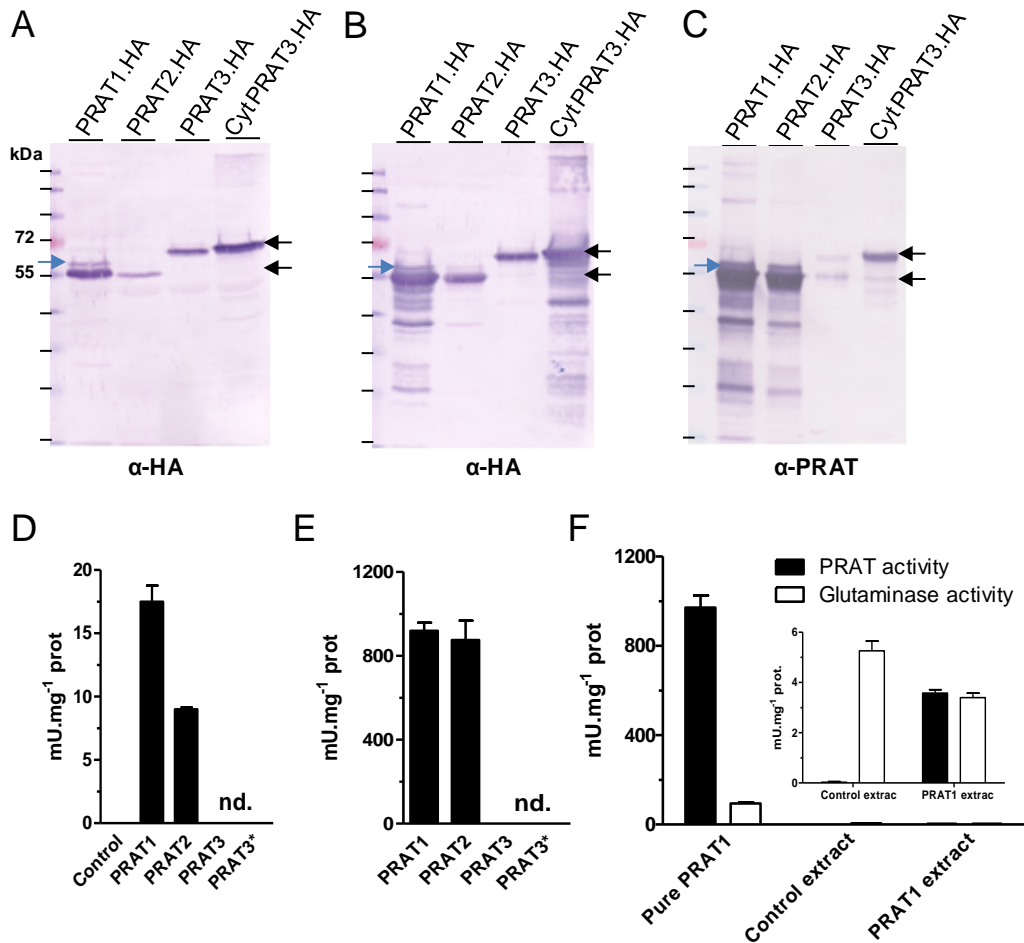
Fig. 6. Overexpression of PvPRATHA.Strep-tagged proteins in leaves from *Nicotiana benthamiana*. Western blot analysis of PvPRAT1, PvPRAT2, PvPRAT3 and PvPRAT3 without the signal peptide, probed with anti-HA monoclonal antibodies in (A) crude extracts of *N. benthamiana* leaves (20µg of total protein) and (B) after purification by affinity binding to Strep-Tactin sepharose (10µL). (C) Western blot of purified PvPRAT1, PvPRAT2, PvPRAT3 and PvPRAT3 without the signal peptide (10µL) probed with anti-PvPRAT1 polyclonal antibodies. (D) PRAT activity in crude extracts of *N. benthamiana* leaves uninfected (control), or infiltrated with the indicated constructs. (E) PRAT activity of purified proteins. (F) PRAT activity (black bars) and glutaminase activity (white bars) of purified PvPRAT1 protein and of crude extracts of *N. benthamiana* leaves uninfected (control) or over-expressing PvPRAT1 protein. Data are means of at least three independent determinations.

As expected, the activity levels in extracts from non-infiltrated plants or from plants infiltrated with an empty vector was much lower or negligible (Fig. 6D).

According to the levels of protein expression, there was a higher PRAT activity in the crude extracts from leaves expressing the PvPRAT1 than in those expressing PvPRAT2 (Fig. 6D). However, once purified, a similar level of specific activity, of around 1 U.mg^{-1}protein, was found for the PvPRAT1 and PvPRAT2 recombinant proteins (Fig. 6E). Likewise, purified PvPRAT3, when assayed in identical amounts compared to the other PRATs did have a negligible level of specific activity, both when expressed with or without its signal peptide, even though the amount of purified proteins was enough to be detected by the anti HA and anti-PvPRAT1 antibodies (Fig. 6B and 6C).

Enzymatic specificity of the pure PvPRAT proteins was confirmed by assaying the glutaminase activity of the samples in absence of PRPP. As shown in Fig. 6F, unspecific glutaminase activity was very low in comparison to PRAT activity in the purified PvPRAT1 protein. In contrast, crude extracts showed a considerable glutaminase activity, present both in the untreated and in the PRAT-agroinfiltrated leaves of *N. benthamiana* plants, while only the PvPRAT expressing tissues showed high levels of PRPP-dependent PRAT activity (Fig. 6F).

6. Biochemical characterization of recombinant PvPRAT proteins

The highly purified enzyme preparations of PvPRAT1 and 2 were used to determine the kinetics parameters of these proteins. As previously shown in Fig. 6E, similar specific activity was obtained for the purified PRAT1 and 2 samples. Likewise, both enzymes exhibited similar kinetic behaviors, with K_m values of 290±40 and 317±59 μM for glutamine and of 48±12 and 39±12 μM for PRPP, for the PvPRAT1 and 2 proteins, respectively (Table 1). Preliminary analysis using crude extracts from nodules indicated that the K_m values of PvPRAT3 were also very similar to those of the other two PvPRAT enzymes (295± 64 μM for glutamine and 41±12 μM for PRPP) although these results would need to be confirmed once active PvPRAT3 could be purified.

Table 1. Kinetic parameters of PvPRAT1 and PvPRAT2 proteins.

Enzyme	Substrate	Km (μM)	Specific activity (μmol.min^{-1}.mg^{-1})	Kcat (s^{-1})	Catalytic efficiency (s.μM^{-1})
PvPRAT1	Glutamine	290.2 ± 40.3	0.919 ± 0.04	230.55	0.794
	PRPP	48.1 ± 11.9			4.79
PvPRAT2	Glutamine	317.0 ± 59.2	0.874 ± 0.09	217.58	0.686
	PRPP	38.7 ± 11.7			5.61

Enzymatic assays in the presence of several nucleotides were done to evaluate the inhibition of PvPRATs by the end products of the *de novo* purine synthesis pathway. As is shown in Table 2, enzymatic activity of highly purified preparation of the PvPRAT1 protein was more highly inhibited by AMP than by IMP and GMP. On the other hand, the addition of either two of three nucleotides did not exhibited a higher level of inhibition than any of them when used alone, thus indicating the absence of a cooperative inhibitory effect (Table 2).

Table 2. Inhibition of PvPRAT1 activity by purine nucleotides.

Nucleotide	Concentration (mM)	Activity (%)
Control	----	100
IMP	2	83.56 ± 6.77
AMP	2	56.73 ± 10.58
GMP	2	91.17 ± 4.97
AMP + IMP	1 + 1	63.04 ± 1.65
IMP + GMP	1 + 1	91.64 ± 8.75
AMP + GMP	1 + 1	62.26 ± 10.72

7. Block of *PvPRAT3* expression affects the ureidic metabolism in common bean grown under N$_2$ fixation conditions

As shown before, biochemical or functional characterization of PvPRAT3 was prevented by the lack of active protein that could be purified from *N. benthamiana* leaves. To gain further functional insights of this protein, an RNAi construct was used to specifically inhibit the expression of *PvPRAT3* gene in

transformed *P. vulgaris* hairy roots. As depicted in Fig. 7A, expression level of *PvPRAT3* was reduced below 50% in hairy roots transformed with the RNAi-*PvPRAT3* construct. Due to the high degree of similarity among the three copies of *PvPRAT* genes, RNAi-silencing of *PvPRAT3* also inhibited the expression of *PvPRAT1*, although the effect was lower (25%) than for *PvPRAT3* (55%). Nonetheless, there was no effect on the basal expression level of *PvPRAT2* in these samples (Fig. 7A). According to the inhibition of *PvPRAT3* expression, a significant reduction in the enzymatic PRAT activity was found in the silenced root samples (Fig. 7B).

RNAi-*PvPRAT3* transformed roots showed a slight reduction of N_2 fixation in comparison with the control roots transformed with an empty-RNAi vector (Fig. 7C). Moreover, *PvPRAT3* silenced roots also contained lower levels of ureides than the roots transformed with the empty vector (Fig. 7D). Since ureides are the main export nitrogen molecules from fixed nitrogen in common bean, the lower level of these compounds in the *PvPRAT3* silenced roots than in the control, transformed with the empty vector, prompted us to search for changes in other metabolites. Metabolomic profile of RNAi-*PvPRAT3* transformed roots in comparison with the control, showed a reduction in adenine and allantoin levels. However, the most prominent change between *PvPRAT3* silenced roots and control ones was a significant increment in the amino acids pool, (Fig. 7E and Supplementary Table S3). As expected from the inhibition of the PRAT activity, content of glutamine, the substrate of the enzyme, increased about 3-fold (Fig. 7F). In addition, significant increments were found for several other amino acids, including asparagine (Fig. 7G). Besides the aminoacids pool, changes were also found in some carbohydrates, as Glu-6-P, glycerol, and galactaric acid, whereas there was a significant reduction in the concentration of pyruvate and an increment in malate and citrate (Fig. 7E and Supplementary Table S3).
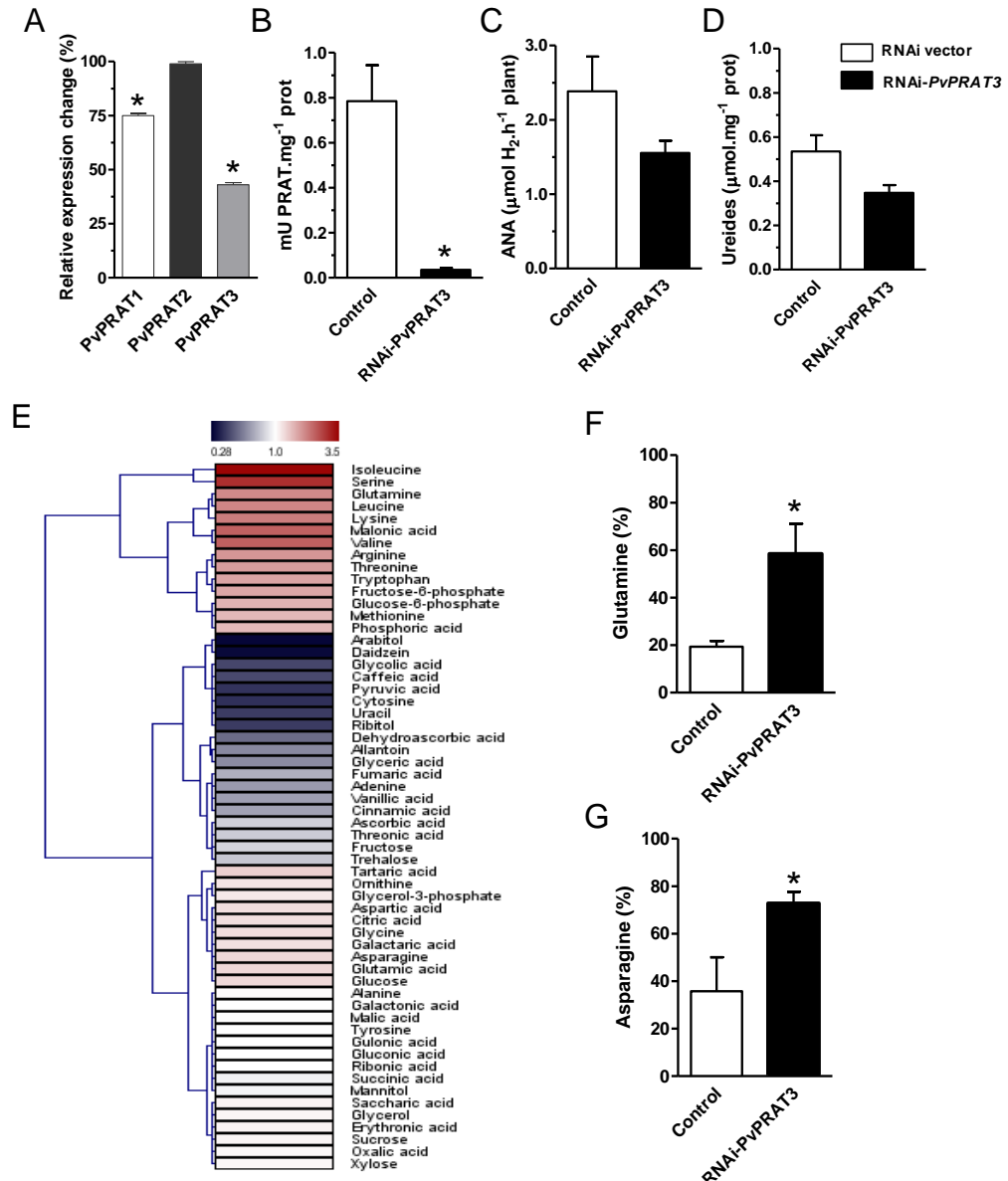
Fig. 7. Effects of *PvPRAT3* mRNA silencing in *P. vulgaris* transformed hairy roots. (A) *PvPRAT1*, *2* and *3* mRNAs expression. (B) PRAT activity. (C) Apparent nitrogenase activity (ANA). (D) Ureide concentration. (E) Clustered heat map of the changes in metabolite pools in RNAi-*PvPRAT3* silenced roots relative to levels in control transformed roots. Colours indicate min-max relative differences between control and *PvPRAT3* silenced roots (dark blue, maximum decrease; dark red maximum increase). (F) Glutamine and (G) Asparagine contents in hairy roots from transformed common bean plants. Hairy roots from *P. vulgaris* plants transformed using *Agrobacterium rhizogenes* carrying empty pFGC5941 vector as control or pFGC5941-*PvPRAT3* silencing construct (RNAi *PvPRAT3*) were analyzed. Data are means of six independent experiments. Significant differences according to Student´s *t*-test are denoted by asterisks (* $p < 0.05$).

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8. PRAT3 is the predominant gene expressed in nodules only in ureidic legumes

The possible implication of PRAT3 in the ureidic behavior of legumes was investigated by comparing the expression levels of *PRAT* genes in tissues from two amidic legumes, *Medicago truncatula* and *Lotus japonicus*, with those of *PRAT*s in *P. vulgaris* tissues. Database searchs revealed the presence of two copies of genes coding putative PRATs in these two amidic legumes, whereas in *Glycine max*, belonging to the ureidic type, there were five PRAT coding sequences (Fig. 8A). Alignment of the protein sequences showed that, as previously depicted in Fig. 2, PvPRAT1 and 2 were more closely related than PvPRAT3. When compared to soybean proteins, PvPRAT3 was closer to the GmPRAT3 than to the PvPRAT1 and 2. Moreover, when comparing with the sequences from the amidic legumes, PvPRAT3 appeared close to the PRAT1 (*Chr1*) from Lotus and to the PRAT1 *(Chr3)* from Medicago, indicating that PRAT3 was present before the separation of the two legume clades. In *Arabidopsis thaliana*, also 3 copies are found, being ATase3 the most distant to ATase1 and 2 but, as well, the less related to the PvPRAT3 proteins.

As shown in Fig. 8B, in contrast to the high expression of *PvPRAT3* in nodules, the expression of either *LjPRAT1* or *MtPRAT1* was only slightly overrepresented in the functional nodules of these amidic plants. In Lotus, *LjPRAT1* was the *PRAT* copy expressed to higher levels, not only in nodules, but also in leaves and roots. In contrast, there were no differences in the expression levels in any of the three tissues among the two gene copies found in Medicago (Fig. 8B). All together, these results indicate that, in contrast to the high expression of *PvPRAT3* in nodules of ureidic legumes, as common bean, there is not a dedicated PRAT gene specifically expressed in nodules of these two amidic legumes.
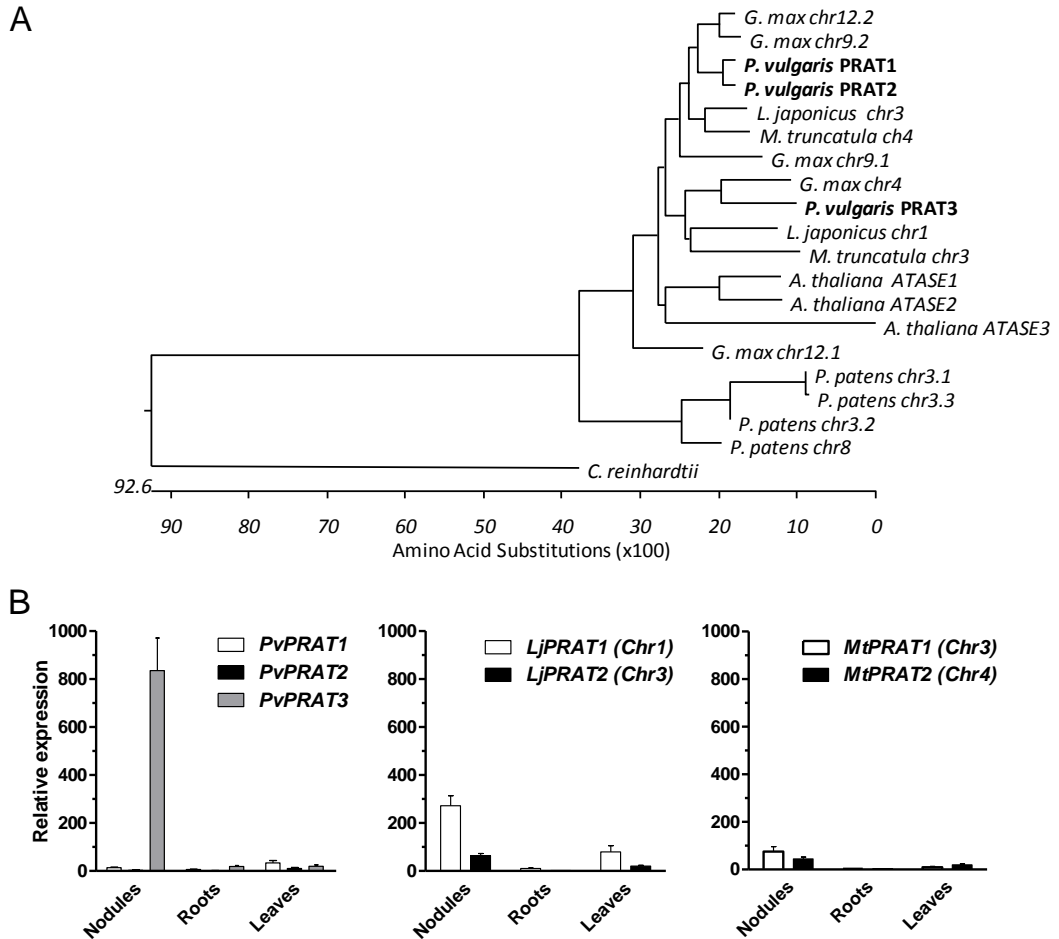
Fig. 8. PRAT proteins in plants. **Expression levels of PRAT genes in tissues of ureidic and amidic legumes.** (A) Phylogenetic distances among PRATs from plants. Sequences were aligned using the Clustal W method and cladogram was constructed by the MegAlign software after multiple amino acid sequence alignment. Sequences were obtained from the Phytozome v10 and miyakogusa.jp[2.5] databases (accession numbers are shown in brackets): *P. vulgaris* PvPRAT1 (Phvul.011G103800), PvPRAT2 (Phvul.011G170200), PvPRAT3 (Phvul.009G002200), *G. max* chr12.2 (Glyma12G036200), *G. max* chr9.2 (Glyma09G2227009), *G. max* chr12.1 (Glyma12G036100), *G. max* chr9.1 (Glyma09G222600), *G. max* chr4 (Glyma04G007300), *L. japonicus* chr1 (chr1 CMO113.320.r[2].d), *L. japonicus* chr3 (chr3 CM0091.2140. r[2].d), *M. truncatula* chr3 (Medtr3g117590), *M. truncatula* chr4 (Medtr4g072110), *A. thaliana* ATase1 (AT2G16570), *A. thaliana* ATase2 (AT4G34740), *A. thaliana* ATase3 (AT4G38880), *P. patens* chr3.1 (Phpat003G120400.1), *P. patens* chr3.3 (Phpat003G120500), *P. patens* chr3.2 (Phpat003G120400.2), *P. patens* chr8 (Phpat008G032200), *C. reinhardtii* (Cre05.g234638). (B) Relative expression levels of *PRAT* genes in amidic and ureidic legumes. qRT-PCR analysis of the expression of *PRAT* gene copies in *M. truncatula*, *L. japonicus* and *P. vulgaris* tissues. Data were normalized to the expression of the plant-specific *Actin* genes.

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DISCUSSION

The *de novo* purine pathway is a highly conserved route consisting of ten enzymatic reactions, catalyzed by ten independent enzymes in bacteria, whereas only six enzymes, including one tri-functional and two bi-functional proteins, are involved in animals, including humans (Smith and Atkins, 2002). In plants, only the last two steps to form the first nucleotide (IMP) are catalyzed by a bifunctional enzyme, the 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase (ATIC), and, therefore, nine proteins in plants carry out the same pathway than microorganisms and animals (Supplementary Fig. S2) (Zrenner *et al.,* 2006).

Glutamine phosphoribosylpyrophosphate amidotransferase (PRAT) is the enzyme that catalyses the first step in purine biosynthesis, the transfer of the amide group from glutamine to PRPP to form 5-phosphoribosylamine (PRA). Whereas nearly all enzymes of the pathway are encoded by single genes in most organisms, PRAT is encoded by three genes in Arabidopsis. This is not unexpected since, Arabidopsis, despite its small genome, is an ancient tetraploid, with two or more copies of the majority of its genes (Initiative, 2000). Search in the available higher plants sequenced genomes shows that, in most non-legumes, PRATs are encoded by two gene copies. Similarly, there are two copies apparently coding PRATs in the amidic legumes *Lotus japonicus* and *Medicago truncatula*. In ureidic legumes as *Phaseolus vulgaris* and *Glycine max*, PRAT is encoded by three and five genes, respectively (Supplementary Table S2). It is tentative to speculate that the larger number of PRAT coding genes in ureidic legumes compared to other plants may be related to the key role of this enzyme for ureides synthesis in their nodules. Soybean is an ancient polyploid with two large-scale genomic duplications that occurred approximately 59 and 13 million years ago (Schmutz *et al.,* 2010). As the most recent duplication event occurred only in soybean, after the separation of the common bean and soybean lineages, it makes sense that almost all gene functions in soybean are represented by twice copies

than those in common bean. From the five PRATs copies in soybean, only the sequence with the highest identity to the gene more highly expressed in common bean nodules, *PvPRAT3*, is represented by a single copy (Fig. 8A), suggesting that the adjustment of copy number is important to maintain the control of purine synthesis in nodules of this ureidic legume.

Noteworthy, the analysis of the genomic organization of *PRAT* genes reveals the lack of spliceosomal intron sequences in the vast majority of the PRAT coding sequences from higher plants (Supplementary Table S2; Data released from *Phytozome* and NCBI databases). In contrast, 10-12 introns are recognized within the genes from animals, including the single copy human gene. Similar intron-rich and single copy *PRAT* genes are found in the moss *Physcomitrella patens* and in the algae *Chlamydomonas reinhardtii*, thus indicating that *PRAT* genes have undergone gene duplication and intron-loss during the evolution of plants. Intronless genes encode proteins from a variety of families with essential housekeeping functions both in animals and plants, although there are a considerable number of plant specific members (Switzer, 1989; Jain *et al.,* 2008). Our work indicates that PRATs should be added to the list of essential genes that evolved as intronless only in higher plants. The origin of intronless genes in eukaryotes is not clear, although it has been suggested that, among other possibilities, intronless evolved as a result of reverse transcription and insertion of the cDNAs into new genomic positions (Wang, 2014; Yan, 2014). It is noteworthy that the only duplication of a PRAT coding sequence reported in animals is, in fact, an intronless PRAT gene found in *Drosophila*, which has been suggested to be originated by a retrotransposition event (Malmanche *et al.,* 2002). The protein sequences deduced for the three *P. vulgaris* cDNAs encoding PRAT, show high similarity with the ATases characterized in *A. thaliana*. They have the two characteristic functional domains of type II glutamine amidotransferases (a glutaminase domain fused to a phosphoribosyltransferase or synthase domain), as well as all the conserved residues that have been described as important for protein function (Zalkin and Dixon, 1992). An N-terminal cysteine has been

proposed to act as a nucleophile residue in the glutaminase portion of the two-step reaction catalyzed by the enzyme (Brannigan *et al.,* 1995). In the enzyme from *Escherichia coli* and yeast, a cysteine is the first residue at the N-terminal, whereas in the *Bacillus subtilis*, chicken and human proteins there is an 11 amino acid propeptide that should be removed to expose the N-terminal cysteine. The three PvPRATs reported here (Fig. 1), as the ATases from *Arabidopsis,* carry also a longer propeptide, with a chloroplast-targeting peptide in addition to the 11 amino acids to be removed to expose the first cysteine (Ito *et al.,* 1994; Kim *et al.,* 1995; Hung *et al.,* 2004). Moreover, PvPRAT proteins contain the Asn-102 and Gly-103 invariable residues that form an oxyanion hole adjacent to the Cys-1 nucleophile, involved in the glutaminase activity (Smith 1995). PvPRATs also have four cysteines at conserved positions proposed as the [4Fe-4S] cofactor-binding residues in *B. subtilis* (Sugiura and Takeda, 2000) and that are also found in PRATs from Arabidopsis and from soybean nodules (Kim *et al.,* 1995).

Analysis of specific expression of the three *PRATs* coding genes in common bean showed than only one of the three copies, *PvPRAT3,* was highly expressed in nodules, exhibiting expression levels over 20-fold higher than those of *PvPRAT1* and *2* genes (Fig. 3A). Differential expression patterns were found for the three ATases in Arabidopsis (Ito *et al.,* 1994; Hung *et al.,* 2004). However, despite the higher relevance of the *de novo* synthesis of purines in ureidic legumes, this is the first report in which the differential tissue-specific expression of *PRAT* genes has been subjected to an in depth analysis in legumes. Results presented here agree with early work on characterization of purine synthesis in moth bean and soybean, in which *PRAT* expression levels in nodules largely exceeded those in leaves (Reynolds *et al.,* 1984; Kim *et al.,* 1995). However, the analysis of the specific expression of each gene was not done in these studies. Recent results at the *P. vulgaris* RNAseq-expression atlas, also showed elevated expression of *PvPRAT3* (Phvul.009G002200) in functional, N_2-fixing nodules (O'Rourke *et al.,* 2014). Our data agree with the expression profiles of the three *PvPRATs* recently uploaded in the *Phytozome* V.10 platform (Schmutz *et al.,* 2014), whilst

experimental data on regulation of the expression of any of the three genes is largely missing. Using qRT-PCR and primers that specifically amplify each of the three *PvPRAT* genes, we have shown that only one, *PvPRAT3*, is highly expressed in nodules, whereas *PvPRAT1* is expressed in leaves, although to considerably lower levels than those reached by *PvPRAT3* in nodules (Fig. 3A and 3B), strongly suggesting that *PvPRAT3* is the copy dedicated to the *de novo* purine synthesis in nodules.

Antibodies against PvPRAT1 detected proteins of the apparent molecular mass of the mature PRAT proteins in nodules, stems and leaves, while in roots a higher apparent molecular mass protein was detected (Fig. 3C), suggesting that the low PRAT specific activity in roots was not only due to low transcripts expression, but also to a lack of an efficient maturation of the protein in these tissues. According to the transcripts expression levels, the highest specific PRAT activity was found in the nodules, followed by activity in leaves. In contrast, roots and stems exhibited low specific activity, although a significant PRPP-independent glutaminase activity was found in these tissues (Fig. 3D). High activity levels in nodules were shown in the early studies on the enzymology of purine biosynthesis in soybean (Schubert, 1981; Boland *et al.,* 1982; Reynolds *et al.,* 1982). In summary, PRAT gene expression, protein and activity in *P. vulgaris* tissues suggest a prominent role for PvPRAT3 in the *de novo* synthesis of purines for the production of ureides in the nodules, whereas PvPRAT1 is the predominant form in leaf chloroplasts, probably generating the purine levels required for plant development. Temporal pattern of *PvPRAT* gene expression in nodules strongly supports our previous results, *PvPRAT3* transcript levels clearly parallel nodule weight and N_2 fixation rate over development (Fig. 4).

Nitrogen availability is known to negatively regulate the concentration of ureides in shoots of nitrate-fed plants (Díaz-Leal *et al*., 2012). We show that nitrate fertilization did not have any relevant effect on the level of expression of *PvPRAT1* or *PvPRAT2* in the leaves. In contrast, there was a lower level of *PvPRAT3* expression in the leaves from plants cultured under nitrate fertilization

in comparison with those under N_2 fixation conditions (Fig. 4C), thus indicating a differential regulation by primary nitrogen assimilation products for this gene copy. The relationship between the products of N_2 fixation and the activity of the enzymes of purine pathway was first reported in studies where N_2 fixation was inhibited by exposure of nodulated roots to an atmosphere of 80% Ar: 20% O_2 (v/v) (Atkins *et al.,* 1984). In these studies, Ar replaces N_2 and thus there is no N_2 fixation, although the nodules remain functional. After this treatment, the purine synthesis, measured as IMP formed, was severely reduced. Analysis of intermediates of the pathway suggested a blockade after the synthesis of formylglycinamide ribonucleotide (GAR) (Supplementary Fig. S2), step 3 of the pathway, suggesting that PRAT activity, at step 1, was unaffected by the blockade in N_2 fixation. Later on, it was demonstrated that transcripts levels, protein abundance and enzymatic activity of the enzymes at step 3 and 5 were affected by the transient inhibition of N_2 fixation (Smith and Atkins, 2002). Our results argue against a lack of regulation by nitrogen source of the first step of purine synthesis, since there was a negative effect of long-time nitrate fertilization in the expression of *PvPRAT3* (Fig. 4C).

Moreover, short-time treatment with sufficient nitrate to inhibit N_2 fixation did inhibit PvPRAT activity in the nodules, while there was not a consistent effect on the transcript level of any of the *PvPRAT* genes (Fig. 5). This result indicates that nitrate exerts its inhibitory effect on purine synthesis mainly by inhibiting PvPRAT3 activity. Interestingly, the inhibition of PvPRAT activity by nitrate is accompanied by the transcriptional down-regulation of uricase, followed by the senescence of the treated nodules (Fig. 5D and 5E). Kim *et al.* (1995) showed that the expression of PRAT (*pur1*) was induced by glutamine, whereas the expression of *pur2* and *pur3* encoding enzymes for step 2 and 3 was unaffected (Schnorr *et al.,* 1996), further supporting that products of N_2 fixation regulate purine synthesis by increasing the level of PRAT. Furthermore, the expression of these genes was unaffected by addition of NH_4NO_3, which agrees with the results presented here for *PRAT* genes after nitrate short-term treatments.

Biochemical characterization of enzymes requires their purification under conditions that preserve protein integrity and activity. In our work, fast and easy purification of the PvPRAT recombinant proteins from *N. benthamiana* leaves, yielded highly purified preparations of active PvPRAT1 and 2 proteins. Unfortunately, sufficient recombinant active PvPRAT3 protein for its biochemical characterization was not obtained (Fig. 6). Specific PRAT activity was higher in the protein extracts from *N. bethamiana* leaves expressing PvPRAT1 than in those expressing PvPRAT2 proteins, according to the lower amount of protein expressed in the latter. However, the specific activity of the two proteins was very similar after their affinity purification, thus indicating that both sequences code for active enzymes with similar biochemical properties, as expected by their almost identical sequences (Fig. 2). Similarly, functional redundancy was also suggested for the *ATase1* and *2* in Arabidopsis (Hung *et al.,* 2004). It is remarkable that the level of transcript expression of *PvPRAT2* gene in the tissues and conditions studied was consistently lower than those of *PvPRAT1* or *3*. Analysis of the 5´UTR of the *PvPRAT* genes revealed an unusual proximity of TATA-box and transcription start sequences in *PvPRAT2* gene (Supplementary Fig. S3), which could has a negative impact in the level of expression of this gene. Nevertheless, alternative explanations, as possible differences in the regulatory sequences in these genes, should also be explored to understand why these two apparently redundant proteins are expressed to different levels in the same plant tissues.

Kinetic parameters of the highly purified PvPRAT1 and 2 revealed that *Km* for glutamine (290 and 317 µM, Table I) is lower than the one determined for ATase2 in Arabidopsis (1.34 mM), and considerably lower that the *Km* for the protein purified from soybean nodules (18 mM), perhaps reflecting the instability of the protein from Arabidopsis and soybean along the purification procedure (Reynolds *et al.,* 1984; Hung *et al*., 2004). Similarly, lower *Km* for PRPP (48 and 38 µM, Table 1) was found for PvPRAT1 and 2 compared to the 0.4 mM determined for the protein from soybean nodules (Reynolds *et al.,* 1984). According to their high

degree of amino acid identity, PvPRAT1 and 2, exhibited similar kinetic parameters, thus further supporting the functional redundancy of the two proteins. End-products inhibition of PRATs has been long recognized as one of the main mechanisms to regulate the *de novo* purine synthesis in bacteria and animals (Caskey *et al.,* 1964; Meyer and Switzer, 1979; Chen *et al.,* 1997a) as well as in plants (Reynolds *et al.,* 1984; Kim *et al.,* 1995; Walsh *et al.,* 2007). Purine nucleotides have been shown to compete with PRPP for the binding site, and two possible sites have been proposed to explain the cooperative inhibitory effect found for adenine and guanine nucleotides (Chen *et al.,* 1997a; Smith 1998). Results presented in Table 2 show that the activity of PvPRAT1 is negatively regulated by the end-products of the pathway, mainly AMP, whereas IMP and GMP were weak inhibitors, consistent with the high concentration of the adenine nucleotide pool found in chloroplasts (Stitt *et al.,* 1982; Wagner and Backer 1992; Zrenner *et al.,* 2006;). No sign of cooperative inhibitory effects were found for the enzyme from *P. vulgaris*, thus arguing against the existence of two possible nucleotide binding sites in this protein.

Expression in *E. coli* of active ATase2 and 3 recombinant proteins was achieved using constructs lacking the chloroplast targeting signal peptide sequences (Walsh *et al.,* 2007). These constructs were designed to contain the 11 residues propeptide, whose autocatalytically removal is essential for glutaminase activity in the *B. subtilis* protein (Mantsala and Zalkin 1984a; Brannigan *et al.,* 1995). However, the authors failed to express recombinant ATase1 with this procedure (Walsh *et al.,* 2007). Using a similar approach, we generated a construct to express *PvPRAT3* without the targeting signal sequence. High expression of the protein lacking the signal peptide was obtained, but surprisingly, the apparent mass of the protein was larger than expected, and there was no significant increase of activity in these extracts (Fig. 6). As for the ATase1 (Walsh *et al.,* 2007), we cannot justify the lack of expression of active PvPRAT3, either with its signal peptide or without it. Nevertheless, it is remarkable that the only protein unable to over-express in plant leaves is the one having the most divergent

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sequence and the one most highly expressed in root nodules. Studies on *B. subtilis* PRAT have shown that its overproduction in *E. coli* leads to the appearance of soluble proenzyme forms as well as the mature enzyme. Moreover the mature form was increased by simultaneous overexpression of the *A. vinelandii nifS,* which is thought to enhance the assembly of the [4Fe-4S] clusters (Chen *et al.,* 1997b). Although the [Fe-S] center does not have a role in catalysis (Vollmer *et al.,* 1983), its correct assembly is necessary for propeptide processing (Grandoni *et al.,* 1989). Moreover, sensitivity of the [4Fe-4S] cluster to oxygen results in loss of native structure and protein turnover in *B. subtilis* protein (Vollmer *et al.,* 1983).

The [4Fe-4S] cluster is presumably present in *P. vulgaris* PRATs. Detailed comparison of amino acid residues in PvPRATs reveals the existence in PvPRAT3 of two extra cysteine residues in the proximity of those believed to act as ligands for the [4Fe-4S] clusters. We speculate that folding and maturation of PvPRAT3 in the nodules might occur under redox conditions differing from those in leaves, and therefore negatively affecting the production of active protein in tissues other than nodules.

Interestingly, recent evidence show that proteins for the *de novo* purine biosynthesis, including PRAT, form a multienzyme complex, the purinosome, which is induced by elevated requirement of purines (An *et al.,* 2008; An *et al.,* 2010a; An *et al.,* 2010b; Baresova *et al.,* 2012; Deng *et al.,* 2012). The presence of purinosomes has not been investigated in plants yet. However, the high purine synthesis rates known to occur in root nodules of ureidic legumes might create a situation similar to that in purine depleted, or highly dividing cancer cells, were enzymes of purine synthesis associate into the purinosomes to improve their catalytic efficiency (An *et al.,* 2008; Verrier *et al.,* 2012). In such a scenario, it would be expected that the coordinated expression of several members of the multienzyme complex may be required for the correct assembly of each protein. We do not have any clear cut explanation for the larger than expected molecular mass of PvPRAT3 protein when expressed in leaves (Fig. 6), although it is

remarkable that a protein band of similar size was also detected in the roots, where very low activity levels were found. This led us to speculate that PvPRAT3 protein could only be correctly folded and processed in the nodules, whereas the incorrectly processed protein may be covalently modified, most likely by ubiquitination or sumoylation, thus transiently increasing its apparent molecular mass. Nevertheless, further experimental results will be required to determine the conditions required to obtain active PvPRAT3 protein.

Inhibition of expression of *PvPRAT3* in transgenic roots leads to a reduction in the levels of ureides, while only slightly reducing their nitrogenase activity (Fig. 7). Moreover, the comparison of metabolic profiles of *PvPRAT3* silenced roots with the control ones revealed the most prominent change affecting to the amino acids pool, with a significant increment in the amounts of 9 amino acids. All together the changes indicate that inhibition of *PvPRAT3* likely provokes C/N imbalance, thus demonstrating that the specific function of this gene in the nodules of the transgenic roots is not compensated by any of the other two *PRAT* copies.

Interestingly, glutamine and asparagine, whose contents increased in the silenced roots, are the predominant compounds of nitrogen transport in most plants, including amidic legumes. Glutamine and asparagine are also the predominant forms of nitrogen export in ureidic legumes, including *P. vulgaris,* when the plants are fertilized with a source of inorganic nitrogen (Díaz-Leal *et al*., 2012). Inhibition of ureide synthesis by the xanthine dehydrogenase (XDH) inhibitor, allopurinol, suggested that fixed nitrogen is not assimilated via alternative pathways, as asparagine synthesis. However, the fast inhibition of N_2 fixation and nodule senescence caused by allopurinol (Atkins *et al.,* 1988) would prevent the assimilation of fixed ammonia into glutamine, also required for the synthesis of asparagine. Results on *PvPRAT3* expression, together with the effects of nitrate fertilization shown in Fig. 4 and 5, suggest that the inhibition of PRAT activity in the nodules of ureidic legumes could be responsible for the increment in glutamine and asparagine that could replace the ureides as main nitrogen export

compounds.

The amidic legumes *L. japonicus* and *M. truncatula* carry two *PRAT* gene copies, but none of them is expressed to high levels in N_2-fixing nodules of the amidic type legumes, despite the higher similarity of one of them with PvPRAT3 than with PvPRAT1 and 2 (Fig. 8). This might suggest that the regulation of the expression of *PvPRAT3* could have evolved for the high rate synthesis of purines to incorporate the fixed nitrogen into ureides only in the ureidic class of legumes. In N_2-fixing nodules, high expression of the purine oxidation enzymes xanthine dehydrogenase (Triplett 1985) and UO (Sánchez *et al.,* 1987) will prevent accumulation of the end-products of the pathway, the nucleotides, and the inhibition of PRAT activity, thus ensuring accumulation of ureides that correlates the N_2-fixation rates in ureidic legumes (McClure *et al.,* 1980; Herridge and Peoples, 1990).

In summary, the three gene copies coding for glutamine phosphoribosylpyrophosphate amidotransferase in *P. vulgaris* encode functional proteins that are expressed differentially in plant tissues. One of the three genes, *PvPRAT3*, is highly expressed in nodules and protein amount and enzymatic activity in these tissues correlate with N_2 fixation activity. PvPRAT1 and 2 proteins have similar kinetic parameters, and show the typical end-product inhibition by purine nucleotides. On the other hand, RNAi-silencing of *PvPRAT3* gene expression and subsequent metabolomic analysis of the transformed roots shows that PvPRAT3 is responsible for *de novo* synthesis of purines used to generate ureides in nodules of ureidic legumes, such as *P. vulgaris*.

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Supplementary material

Supplementary Fig. S1. Southern-blot of *PRAT* genes from *Phaseolus vulgaris*.

Supplementary Fig. S2. *De novo* plant purine synthesis pathway.

Supplementary Fig. S3. Schematic representation of the 5´UTR sequences of *PvPRAT* genes.

Supplementary Table S1. List of primers used in this study.

Supplementary Table S2. Number of PRAT coding sequences present in the genome of some representative organisms.

Supplementary Table S3. Comparison of metabolomic profiles of roots transformed with a *PvPRAT3* RNAi vector to or control profiles.
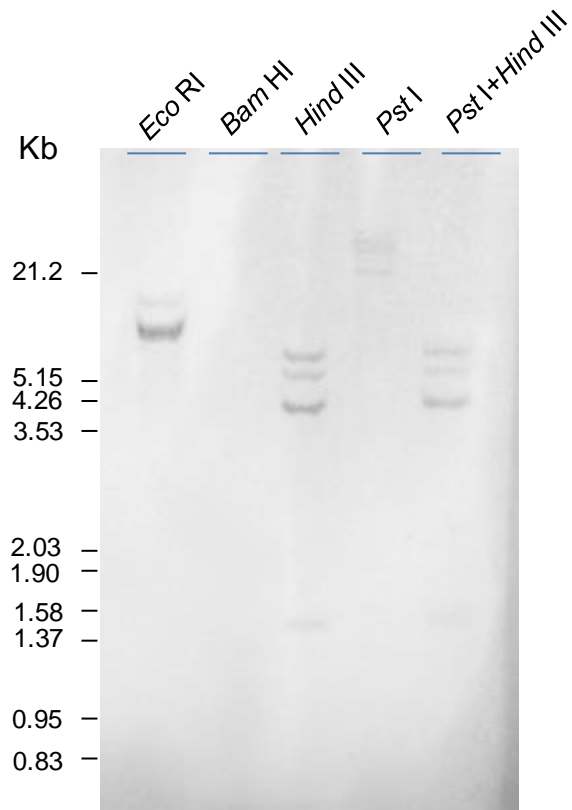
Supplementary Fig. S1. Analysis by southern-blot of *PRAT* genes from *Phaseolus vulgaris*.

Glutamine (**GLN**) **+** 5-phospho-D-ribosyl-1-pyrophosphate (**PRPP**)

1   ↓   *Phosphoribosylpyrophosphate amidotransferase (**PRAT**)*
              EC 2.4.2.14

5-phosphoribosylamine (**PRA**)

2   ↓   *GAR synthase (**GARS**)*
              EC 6.3.4.13

5-phosphoribosylglycinamide (**GAR**)

3   ↓   *GAR transformylase (**GART**)*
              EC 2.1.2.2

5-phosphoribosyl-N-formylglycinamide (**FGAR**)

4   ↓   *FGAM synthase (**FGAMS**)*
              EC 6.3.5.3

5-phosphoribosyl-N-formylglycinamidine (**FGAM**)

5   ↓   *AIR synthase (**AIRS**)*
              EC 6.3.3.1

5-phosphoribosylaminoimidazole (**AIR**)

6   ↓   *AIR carboxylase (**AIRC**)*
              EC 4.1.1.21

1-(5-phosphoribosyl)-5-amino-4-carboxyimidazole (**CAIR**)

7   ↓   *SAICAR synthase (**SAICARS**)*
              EC 6.3.2.6

1-(5-phosphoribosyl)-4-(N-succinocarboxamide)-5-aminoimidazole (**SAICAR**)

8   ↓   *Adenylosuccinate lyase (**ASL**)*
              EC 4.3.2.2

1-(5-phosphoribosyl)-5-amino-4-imidazolecarboxamide (**AICAR**)

9   ↓   *AICAR transformylase/IMP cyclohydrolase (**ATIC**)*
              EC 2.1.2.3   EC 3.5.4.10

1-(5-phosphoribosyl)-5-formamide-4-imidazolecarboxamide (**FAICAR**)

10   ↓   *AICAR transformylase/IMP cyclohydrolase (**ATIC**)*
              EC 2.1.2.3   EC 3.5.4.10

Inosine monophosphate (**IMP**)

Supplementary Fig. S2. *De novo* plant purine synthesis pathway**.** The enzymes participating in the ten enzymatic reactions and the pathway intermediates are shown. Step 9 and 10 are catalyzed by a single bifuntional enzyme (Aminoimidazole carboxamide ribotide (AICAR) transformylase and inosine monophosphate (IMP) cyclohydrolase) in plants (adapted from Zrenner *et al.,* 2006).

Supplementary Fig. S3. Schematic representation of 5´UTR sequences of *PvPRAT* genes. Transcription start site indicated with an arrow. Positions of CAAT and TATA boxes are shown.

Supplementary Table S1. List of primers used in this study. Restriction sites of enzymes used for cloning are underlined.

Primer use	Gene	Primers sequences (5´- 3´)
RACE-PCR	*PvPRAT1*	GSP1 (GGTGGTGTTGAAGATGGAG)
		GSP2 (TCGTCGGCATCTATGGCGA)
	PvPRAT2	GSP1 (TCACTCATTTCAATCTCAGAC)
		GSP2 (GAAAGTGAGAACATCGGAGAG)
	PvPRAT3	GSP1 (AGAATCGTTGACGCCTGCGA)
		GSP2 (TCTCCTTCCCATCACCAAAGG)
Southern probe	*PvPRAT1*	FW (TGATCGAAGCGACATACGAG)
		REV (CAATCTTGGACGAGGTGGTT)
pET30b+ cloning	*PvPRAT1*	FWR (AAGG<u>CCATGG</u>ACGACAAGCCGCGCGA)
		REV (ATAGTTTA<u>GCGGCCGC</u>ACTACCAACCTTGATTTGG)
pXCS-HaStrepII cloning	*PvPRAT1*	FWR (CC<u>ATCGAT</u>CCATGGCCGCAG)
		REV (TCC<u>CCCGGG</u>ACTACCAACCTTGATTTGGT)
	PvPRAT2	FWR (CCC<u>ATCGAT</u>CCATGGCCGCAGCAACACCA)
		REV (ATC<u>CCGGG</u>AAACCGCACTTTCGCTTGA)
	PvPRAT3	FWR (CCC<u>ATCGAT</u>ATGTGCACCAAAGCCACAAACAC)
		FWR-PS (CCC<u>ATCGAT</u>CCATGGATGATGATGACGAAAAGCC)
		REV (AT<u>CCCGGGG</u>ACACTAACAATCTTATCCTCA)
pFGC5941 cloning	*PvPRAT3*	FWR (GC<u>TCTAGA</u>GGCGCG<u>CC</u>GAGTGAAGCTTAAGCTGT)
		REV (CA<u>GGATCC</u>ATT<u>TAAAT</u>AGGCTCGTGGTTTCACGT)
qRT-PCR	*PvAct-2* (KF033666.1)	FWR (GGAGAAGATTTGGCATCACACGTT)
		REV (GTTGGCCTTGGGATTGAGTGGT)
	LjAct-2 (EU195536.1)	FWR (CAGCATTGTTGGTCGTCCTCGTC)
		REV (ACTGTGCCTCATCCCCAACATAG)
	MtAct-2 (JQ028731.1)	FWR (CTTCAATGTGCCTGCCATGTATGT)
		REV (GACTCACACCGTCACCAGAATCC)
	Pv18S (CV670768.1)	FWR (GACACGGGGAGGTAGTGACAAT)
		REV (ACGAGCTTTTTAACTGCAACAACT)
	PvUO (PVU72663.1)	FWR (TCTCTTCTCTGATTGCGTCAACTCGTA)
		REV (CCTTTGCTTTTGCATACACCGTGT)
	PvPRAT1	FWR (GTGAGAGATAATGTCGGTCAAACCATG)
		REV (CATGCATAACCGGCCTTCAGAATCCA)
	PvPRAT2	FWR (TCAAGCGAAAGTGCGGTTTTAGTCAG)
		REV (GTTCCATTCCAAGATGCATCAATCCA)
	PvPRAT3	FWR (GTGGATGTTCGCCATTGGGTTGAG)
		REV AGCAGGGCGGTTTGAGAATCCAGAG

Cont. Supplementary Table S1

qRT-PCR	*LjPRAT1* (chr1.CM0113.320.r2.d)	FWR (ACCCTTCACCAACACACCTTGTTTTC)
		REV (TGGTGTTGTGGTTTTTGCATGGTGATG)
	LjPRAT2 (chr3.CM0091.2140.r2.d)	FWR (TCCTCCTCCTACCACCACCCGCT)
		REV (TGGCGTTGTTGGAGGAGACAGCGT)
	MtPRAT1 (XM003607037.1)	FWR (ACTTAGCCCTCCACGCTCTCCAAC)
		REV (ACCAACACCGGTGATGGATTGAAGA)
	MtPRAT2 (XM003603966.1)	FWR (GATGACGCTCCTTCTTCTTCCATTCCA)
		REV (TGGCCTGCAGTTGAATAACGGATGT)

Supplementary Table S2. Number of PRAT coding sequences present in the genome of some representative organisms. The number of introns found in each gene is indicated. Information retrieved from NCBI or *Phytozome* v10.1 platforms. * Identified in this work.

Organism	Gene copies	Accession	Nº introns
Phaseolus vulgaris	3	Phvul.011G103800	0
		Phvul.011G170200	0
		Phvul.009G002200	0
Glycine max	5	Glyma09G222600	0
		Glyma09G2227009	0
		Glyma12G036200	0
		Glyma12G036100	2
		Glyma04G007300	0
Lotus japonicus	2	chr1 CM0113.320.r^2.d	1*
		chr3 CM0091.2140.r^2.d	0*
Medicago truncatula	2	Medtr3g117590	3
		Medtr4g072110	0
Arabidopsis thaliana	3	(ATASE1) AT2G16570	0
		(ATASE2) AT4G34740	0
		(ATASE3) AT4G38880	0
Oryza sativa	3	LOC_Os05g35580	0
		LOC_Os01g65260	0
		LOC_Os06g39440	0
Physcomitrella patens	4	Phpat003G120400.1	5
		Phpat003G120500.1	4
		Phpat003G120400.2	3
		Phpat008G032200.1	4
Chlamydomonas reinhardtii	1	Cre05.g234638	12
Saccharomyces cerevisiae	1	AAA34403	0
Bacillus subtilis	1	BAM49569	0
Escherichia coli	1	WP000334229	0
Drosophila melanogaster	2	NP729191	3
		NP523949	3
Gallus gallus	1	NP00100440	10
Homo sapiens	1	NP002694	10

Supplementary Table S3. Comparison of metabolomic profile of roots transformed with RNAi-*PvPRAT3* or with empty vector. Compounds showing significant ($p< 0.05$) differences in the RNAi-*PvPRAT3* roots with respect to the control roots (transformed with the empty vector) are highlighted. In red, metabolites showing significantly higher concentration in the *PRAT3* silenced roots than controls are shown; in blue, compounds found at significantly lower concentration. Compounds with no significant differences are shown in white. Data are mean from 6 independent roots.

Metabolite group	Metabolite name	RNAi-*PvPRAT3* roots / empty vector transformed roots
Amino acids	Alanine	1.04
	Arginine	2.03
	Asparagine	1.38
	Aspartic acid	1.32
	Glutamic acid	1.38
	Glutamine	2.15
	Glycine	1.33
	Isoleucine	3.45
	Leucine	2.19
	Lysine	2.28
	Methionine	1.70
	Ornithine	1.27
	Serine	3.04
	Threonine	1.97
	Tryptophan	1.90
	Tyrosine	1.01
	Valine	2.56
N-compounds	Adenine	0.71
	Allantoin	0.66
	Cytosine	0.41
	Uracil	0.45
Sugars	Fructose	0.88
	Glucose	1.37
	Sucrose	1.11
	Trehalose	0.84
	Xylose	1.08
Phosphates	Fructose-6-phosphate	1.87
	Glucose-6-phosphate	1.74
	Glycerol-3-phosphate	1.23
	Phosphoric acid	1.68
Polyols	Glyceric acid	0.67
	Arabitol	0.30
	Daidzein	0.30
	Glycerol	1.11
	Mannitol	0.96
	Ribitol	0.45
Polyhydroxy acids	Ascorbic acid	0.86
	Dehydroascorbic acid	0.58
	Erythronic acid	1.12
	Galactaric acid	1.31
	Galactonic acid	1.01
	Gluconic acid	1.00
	Glyceric acid	0.67
	Gulonic acid	0.58
	Ribonic acid	1.00
	Saccharic acid	1.13
	Threonic acid	0.86

Cont. Supplementary Table S3

Acids	Citric acid	1.33
	Fumaric acid	0.77
	Glycolic acid	0.48
	Malic acid	1.01
	Malonic acid	2.55
	Pyruvic acid	0.42
	Oxalic acid	1.08
	Tartaric acid	1.47
	Succinic acid	0.96
Phenylpropanoids	Caffeic acid	0.49
	Cinnamic acid	0.74

CHAPTER 5: TRANSCRIPTIONAL REGULATION OF *PvPRATs* DURING SEED GERMINATION AND UNDER STRESS CONDITIONS

Abstract

In ureidic legumes, a high rate of ureide synthesis takes place when plants are fixing atmospheric N_2. However, an elevated synthesis of ureides is also observed in ureidic legumes in situations other than N_2 fixation, such as during stress and germination. Until now, the exact origin of these ureides and how their synthesis is regulated is largely unknown. This work has focused on the study of the regulation of the enzyme PRAT from *Phaseolus vulgaris* by these physiological situations. The *in silico* analysis of the proximal promoter sequences of the three *PvPRAT* genes showed that they have several motifs of regulation by stress and by developmental processes, although only the expression of *PvPRAT3* changed under some stress-related conditions, like the treatments with ABA, SNP and ethylene. In a similar manner, the expression of *PvPRAT3* was up-regulated after germination in seedlings at the same time in which the increment in the levels of ureides was observed. However, in plants subjected to drought stress, neither the expression nor the activity of PvPRATs underwent significant changes; thus suggesting that the enzyme and, therefore, the *de novo* synthesis of purines, has low relevance for the synthesis and accumulation of ureides observed when plants are subjected to water deficit.

Introduction

Ureide synthesis is highly activated in nodules of the ureidic legumes, since most of the fixed nitrogen is used to form the ureides allantoin and allantoate, through the oxidation of the purines *de novo* synthesized (Kim *et al*., 1995). In *Phaseolus vulgaris*, the *de novo* synthesis of purines takes place mainly by the activity of *PRAT3* isoform, which is differentially up-regulated in nodules (Chapter 4, Fig. 3). Apart from the N_2 fixation in nodules, there are other physiological situations in which an induction on the synthesis of ureides has been observed. As previously described (Chapter 2), drought stress leads to an induction of ureide synthesis in leaves from *P. vulgaris*, which was higher in the most drought sensitive genotypes than in the tolerant ones. The accumulation of ureides under these situations continued after the N_2 fixation had already ceased due to the imposed stress. Moreover, it was also observed in plants fertilized with nitrate and, therefore, lacking any N_2 fixation, which indicated that the source of ureides could not be the nitrogen fixed and assimilated in the nodules (Alamillo *et al*., 2010). The enhancement of ureide synthesis under water stress could be explained as a part of a general response to stress, where ureides may play both a role in cell protection under oxidative stress conditions and a role in nitrogen mobilization and recycling from either protein or nucleic acid turnover in leaves (Alamillo *et al*., 2010), although the exact source of the ureides remains unclear.

Besides the accumulation under drought stress, increments in the concentration of ureides have been also observed at early stages of germination in soybean and common bean seeds, respectively (Polayes and Schubert, 1984; Quiles *et al*., 2009). Polayes and Schubert (1984) demonstrated that these ureides came from the oxidation of purine nucleotides *de novo* synthesized in cotyledons and that, once formed, were transported to the embryonic axes. Later on, at the beginning of cotyledon senescence, the embryonic axes acquire the capacity to synthesize ureides from *de novo* formed purines and transport them to the developing leaves (Quiles *et al*., 2009).

The regulation of *de novo* purine synthesis, specifically of PRAT protein, during germination and under stress conditions, has not been studied yet. In this work, the presence of possible regulatory sequences in the promoters of *PvPRAT1*, *2* and *3* genes have been *in silico* analyzed and the relative contribution of each PvPRAT isoform to the increment of ureide concentration observed in response to drought-stress and during germination of seeds has been measured.

Materials and methods

1. Plant material, growth conditions and treatments

Seeds of *Phaseolus vulgaris* L. genotypes PHA-0683, Great Northern and PHB-0285 were surface-sterilized by sequential dipping in ethanol (30 s) and 0.2% (w/v) sodium hypochlorite (5 min) and then washed thoroughly with distilled water. Soaked seeds were allowed to germinate in Petri dishes (120 mm diameter) with wet paper under sterile conditions. For the seedling experiments seeds of the Great Northern variety were sterilized and allowed to germinate as is described above. Epicotyl, radicle and cotyledons were collected after 1, 2 and 4 days after imbibition. For the rest of experiments, 4 seedlings were sown on each pot (16 cm diameter, 18 cm height) filled with an artificial substrate composed of vermiculite/perlite mixture (2/1 w/w) and inoculated with a fresh suspension of *Rhizobium leguminosarum* ISP 14, which had been cultured at low temperatures (28 °C) for less than 30 hours. Plants were cultured in growth chamber under a long-day photoperiod (16 hours light, 8 hours dark), 300 $\mu E.m^{-2}.s^{-1}$ lighting, and 70% relative humidity at 26-21 °C (day-night temperatures). Inoculated plants were watered three times a week with nitrogen-free nutrient solution (Rigaud and Puppo, 1975).

Water stress treatment was performed in plants of PHA-0683 (Tolerant), Great Northern (Intermediate) and PHB-0285 (Sensitive) genotypes (Riveiro, 2012) as previously described in Chapter 2. Briefly, at the onset of flowering, which happened at 25–28 days after sowing, (according to bolting dates of each variety),

plants were randomly separated into two sets. Watering was withheld from one half of the plants and the second half was regularly irrigated. Plant collection was done at 7 days from the beginning of the treatment in both, control and water-stressed plants. For phytohormone treatments, leaves from plants at 26 days after sowing of the Great Northern genotype were treated for 48 hours with either 10 µM abscisic acid (ABA), 50 µM jasmonic acid (JA), 200 µM salicylic acid (SA), 2 µM 1-aminocyclopropane-1-carboxylic acid (ACC), 2 µM gibberellic acid (GA) or 200 µM sodium nitroprusside (SNP). Stock solutions were prepared in 2 mL of ethanol (ABA, JA, GA) or 2 mL of distilled water (SA, ACC, SNP), and diluted with the nitrogen-free nutrient solution to achieve the final working concentrations. Leaves were sprayed twice, 48 and 24 hours before its harvest. Control plants were treated with distilled water or with ethanol at the same concentration at the one of each phytohormone in working solutions. All compounds were purchased from Sigma-Aldrich. For high (39ºC) temperature treatment, leaves from plants at 28 days after sowing of the Great Northern genotype were detached and incubated at 39 ºC for 2 hours Detached leaves used as a control were incubated in Petri dishes (120 mm diameter) on moist paper to prevent dehydration, during the same time.

Plant material was collected at indicated times after the treatments, frozen with liquid nitrogen and stored at -80 ºC until further analysis.

2. *PvPRAT*s promoter sequences isolation

The proximal promoter sequences of *PvPRAT1* and *PvPRAT3* genes were isolated using the *GENOME WALKER^{TM} Universal kit* (Clontech). The transcription start sites of *PvPRAT1*, *2* and *3* were determined by the isolation of the 5´-UTR sequence with the *SMART^{TM}RACE* kit (*Rapid Amplification of cDNA-ends*, Clontech). Gene inner specific primers nearly to 5´ end (Supplementary Table S1) were designed according the *PvPRAT* cDNA sequences cloned previously from the Great Northern genotype. Two consecutive PCRs were done, according to manufacturer´s instructions. The resulting DNA fragments were cloned into

pGEMt-easy vector (Promega, Madison WI) or pSparkI vector (Canvax Biotech SL) and the sequence from several independent clones was determined. The identity of the sequences obtained was confirmed by the recently released whole genome sequences from *P. vulgaris* (http://phytozome.jgi.doe.gov/) (Schmutz *et al*., 2014). The promoter sequence of *PvPRAT2* was not isolated due to the releasing of the genome sequence previously to the approach of its cloning and the sequence found at the database was used for the *in silico* analysis.

3. Gene expression analysis

Total RNA was isolated from the different tissues using the TRI REAGENT (Sigma-Aldrich) following the manufacturer's instructions. Prior to RT-PCR, total RNA from control and treated tissues was treated with RNAase-free DNAaseI (Promega, Madison WI) at 37 ºC for 30 min to eliminate any traces of genomic DNA. Lack of 18S rRNA amplification was used to check the successful removal of DNA. First strand cDNA synthesis was done with 2.5 µg of DNAase-treated RNA using iScriptP^{TM} reverse transcriptase (Bio-Rad) following the manufacturer's instructions. Expression analysis was performed by quantitative RT-PCR in an iCycleriQ System (Bio-Rad, Hercules, CA, USA) using the iQ SYBR-Green Supermix (Bio-Rad) and *PvPRAT1*, *2* and *3* specific primers (Supplementary Table S1). The PCR programme consisted of an initial denaturation and Taq polymerase activation step of 5 min at 95 ºC, followed by 50 cycles of 15 s at 95 ºC and 1 min at 60 ºC. Expression of *Actin-2* and *18S* genes was used as internal control, after checking that its level of expression was similar in all samples. Specificity of primers was confirmed by dissociation curves analysis and by sequencing of the PCR products. The amplification efficiency of each primer pairs, calculated by PCR using serial dilutions of root and leaf cDNAs, was > 98%. Analysis of relative gene expression was calculated from ΔCT values (Livak and Schmittgen, 2001), using expression of *Actin-2* gene for data normalization. All the reactions were set up in triplicate (three technical

replicates) using three to five RNA preparations from tissues collected from independent experiments (biological replicates).

4. PRAT activity assay

For the enzymatic assay of PRAT activity, a slightly modification of the method described by Walsh *et al*. (2007) was used. This method is based in the continuous spectrophotometric determination of the NADH produced in a reaction mix in which glutamate dehydrogenase (GDH) activity was coupled to the PRAT-released glutamate. All procedures for protein extracts preparation and enzyme manipulations were carried out at 0–4 °C. Frozen plant material was ground to a fine powder under liquid nitrogen. Plant extracts were obtained by adding 3 mL of extraction buffer per gram of tissue. The extraction buffer for PRAT assay was 100 mM Tea-NaOH, pH 8.0; 150 mM NaCl; 15 mM DTT; 1 mM $MgCl_2$ and 0.5% (w/v) Triton X-100. The resulting homogenate was centrifuged at 15000 *g* for 10 min and the supernatant was dialyzed through *SpinTrap G25* columns previously equilibrated with 100 mM Tea-NaOH, pH 8.0; 5 mM DTT and 1 mM $MgCl_2$ buffer, and the flow-through was used as crude extract.

The enzymatic assay was carried out at 30 ºC, in a reaction mixture composed of 100 mM Tea-NaOH, pH 8.0; 5 mM DTT; 1 mM $MgCl_2$; 1 mM PRPP; 8 mM glutamine; 6 mM NAD^+; 1U GDH and 5% (v/v) of the previously dialyzed crude extract. PRAT activity was determined following the continuous production of NADH by GDH-coupled assay during 5 min. In parallel, we carried out all the measurements without the substrate PRPP, as a control of unspecific glutaminase activity. Soluble protein was measured in the same extracts in which the enzymatic activity was determined (Bradford, 1976).

The results are expressed as means of the values from at least three independent experiments. Enzymatic assays and analytical determinations from each biological experiment were done at least twice (two independent samples) and each assay was measured by duplicate (technical replicate).

5. Statistical analysis of the data

Student´s t-test statistical analysis was performed using the GraphPad software package.

Results

1. Effect of stress-related molecules on *PvPRAT* gene expression

The *in silico* analysis of the proximal sequences upstream of the transcription start of each *PvPRAT* gene was done using the PlantCARE prediction software (Lescot *et al*., 2002). Prediction showed the presence of a high variety of regulatory motifs related with development and stress (Fig. 1A and Supplementary Fig. S1). In the *PvPRAT1* promoter sequence, two TC-rich repeats, a motif related with defense and stress responsiveness, one related with response to drought (MBS), one to JA (CGTCA, TGACG), two related with SA response (TCA-element) and one motif of regulation by GA (GARE) were found. There were also two motifs of endosperm expression (Snk-1 motif) and nine different motifs related to light responses, including one of regulation by circadian rhythm. In the *PvPRAT2* promoter sequence, a lower variety of regulatory motifs than in *PvPRAT1* promoter was identified. They were two TC-rich repeats, two motifs of ethylene response (ERE) and three motifs of high temperature response (HSE). Moreover, in this promoter there was found the highest number of motifs of regulation by light, which were spread along the sequence (Supplementary Fig S2). In the promoter region of the *PvPRAT3* gene, there were found four Snk-1 motifs and one GARE-motif, as well as two TCA-elements, one motif of regulation by ABA (ABRE) and one of anaerobic induction (ARE). As in the promoter sequences of *PvPRAT1* and *2*, in that of *PvPRAT3* gene there were found a number of motifs related to regulation by light, which included two sequences of circadian rhythm regulation (Fig. 1).

As a preliminary evaluation of the functionality of the *cis*-regulatory motifs, the expression of *PvPRAT1*, *2* and *3* genes in response to treatments related with the

motifs found was determined (Fig. 1B). Leaves from Great Northern genotype were treated either with abscisic acid (ABA), jasmonic acid (JA), salycilic acid (SA), 1-aminocyclopropane-1carboxylic acid (ACC), gibberellic acid (GA) and the NO-donor sodium nitroprussiate (SNP), or incubated at 39 ºC. As shown in Fig. 1B, only the expression of *PvPRAT3* gene was regulated by some of the treatments applied. Transcriptional levels of *PvPRAT1* and *PvPRAT2* did not show significant changes in the leaves subjected to the different treatments. In contrast, the transcription of *PvPRAT3* gene showed a significant increment in the leaves treated with ABA, SNP and ACC. Nevertheless, despite the increments in *PvPRAT3* expression, there were no consistent changes in the activity levels of the treated samples, most likely due to the low sensitivity of the PRAT activity assay in leaves.
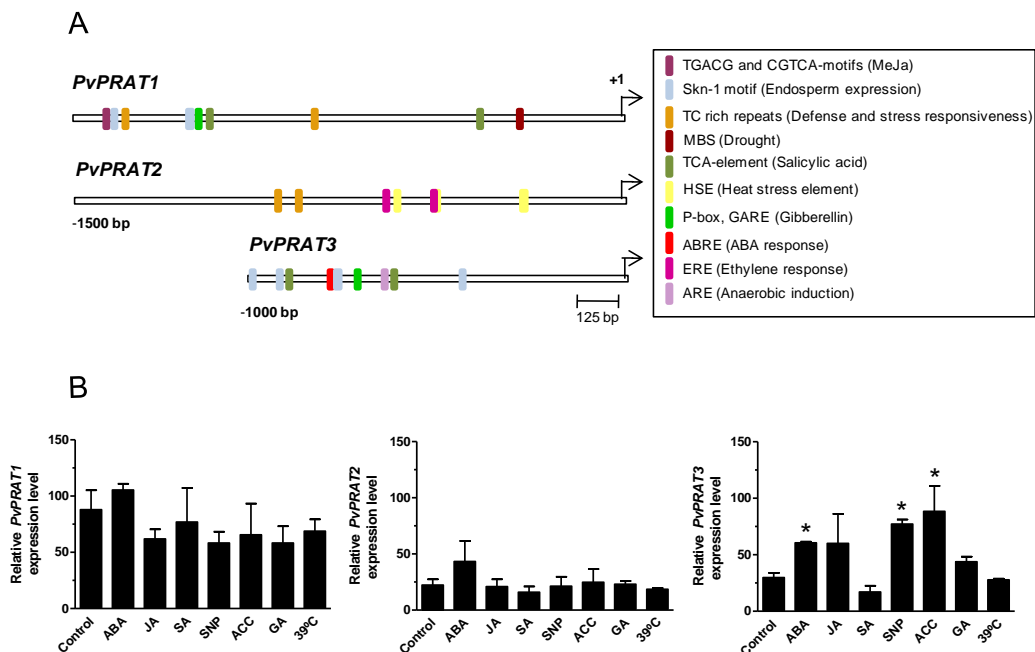
Fig. 1. Expression of *PvPRAT* genes after stress-related treatments. (A) Schematic representation of the *cis*-regulatory elements predicted in the proximal promoter sequences of *PvPRAT1*, *2* and *3* genes. (B) Relative transcript expression of *PvPRAT1*, *2* and *3* in leaves from *P. vulgaris* treated with phytohormones (ABA, JA, SA, SNP, ACC and GA) and submitted to high temperature (39 ºC). Expression data were normalized to *Actin-2* gene expression. Results are representative of at least three independent experiments. Significant differences according to Student´s t-test are denoted by asterisks (* $p < 0.05$)

2. Regulation of PvPRATs during drought stress

PRAT expression and activity levels were analyzed in plants of PHA-0683 (Tolerant), Great Northern (Intermediate) and PHB-0285 (Sensitive) genotypes subjected to water deficit during 7 days (Fig. 2).
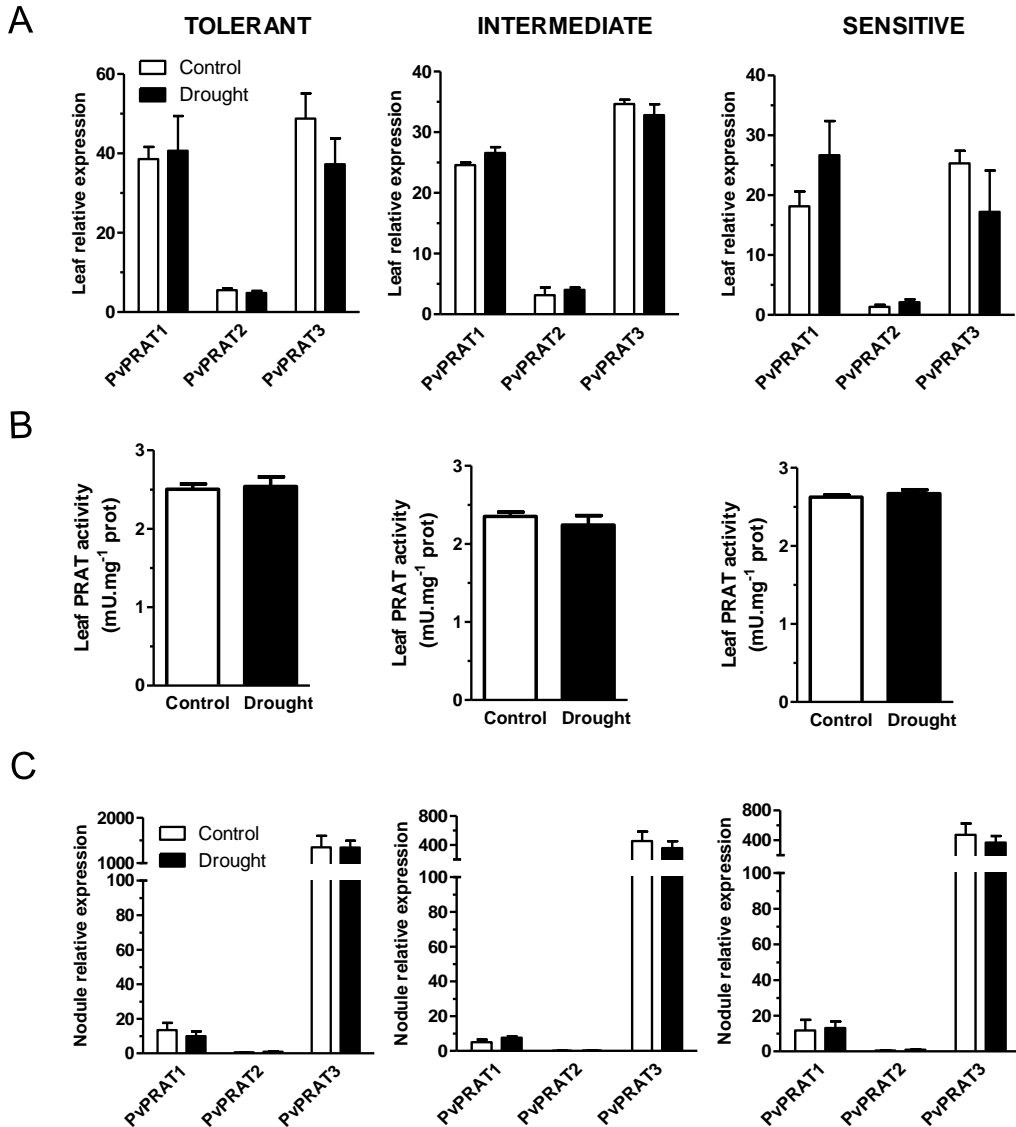
Fig. 2. **PvPRAT response to drought stress.** (A) Relative transcript expression of *PvPRAT1*, *2* and *3* in leaves. (B) Specific PRPP-dependent PRAT activity in leaves. (C) Relative transcript expression of *PvPRAT1*, *2* and *3* in nodules. All the measurements were done in tissues of the tolerant, intermediate and sensitive genotypes after 7 days of water deprivation. Expression data were normalized to *Actin-2* gene expression. Results are representative of three independent experiments.

The degree of drought-tolerance of the three varieties was previously analyzed by Riveiro (2012). PHA-0683 was the genotype which showed the highest resistance to drought stress, Great Northern variety showed an intermediate resistance level and the PHB-0285 was the most sensitive one. In the leaves of the drought-stressed plants, ureide levels increased correlating with the degree of sensitivity of N_2 fixation to water deficit (Chapter 2, Fig. 3). However, neither the expression of *PvPRAT* genes nor the PRAT activity changed in the leaves of any genotype after 7 days of water deficit (Fig. 2A and 2B). Similarly, in the nodules of drought-stressed plants, mRNA level for *PvPRAT*s did not change in comparison with the control nodules in any of the three genotypes (Fig. 2C). This result was in accordance with the absence of changes in the concentration of ureides in the nodules, described previously in Chapter 2 and in Alamillo *et al*. (2010).

3. Induction of *PvPRAT3* during early germination

The concentration of ureides increases during germination of *P. vulgaris* seeds, which takes place between the second and the third day after imbibition (Quiles *et al*., 2009). To elucidate whether the ureides accumulated are products of the oxidation of purines synthesized *de novo*, the expression of Pv*PRATs* was determined in embryonic axes and cotyledons from the Great Northern variey at 1, 2 and 4 days after imbibition. As is shown in Fig. 3, the expression level of *PvPRAT* genes was much higher in cotyledons than in embryonic axes during the whole experimental time. At the first day after imbibition, the mRNA level of *PvPRAT1* and *2* in embryonic axes and cotyledons was higher than that of *PvPRAT3* gene. However, there was a gradual increase in the mRNA level of *PvPRAT3*, whereas the expression levels of *PvPRAT1* and *2* did not change along the experimental time. At the fourth day after imbibition, *PvPRAT3* had reached similar expression levels than *PvPRAT1* and *2* in embryonic axes and was 4-fold higher in cotyledons (Fig. 3).
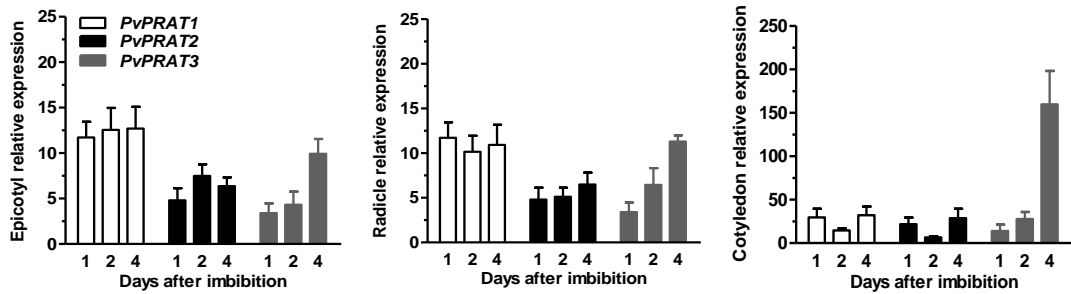
Fig. 3. Expression of *PvPRAT* genes during early seedling development. Relative mRNA expression of *PvPRAT1*, *2* and *3* in axes and cotyledons from *P. vulgaris* at 1, 2 and 4 days after imbibition. Expression data were normalized to *Actin-2* gene expression. Results are representative of three independent experiments.

Discussion

The regulation of the *de novo* purine synthesis, where PRAT performs a key regulatory step, is not yet well understood in higher plants. Most studies of purine biosynthesis in plants have focused on its function in the assimilation of the nitrogen fixed in nodules of ureidic legumes, and the information about its role in normal plant physiology is scarce (Hung *et al*., 2004). Contrary to animals, *PRAT* gene is encoded by multigene families in higher plants (Ito *et al*., 1994; www.phytozome.net) which prompt to suggest that each gene copy might be differentially regulated to probably perform isoform-specifics functions. The analysis of the promoter sequences done in this study showed that each *PRAT* gene from *Phaseolus vulgaris* might be subjected to specific transcriptional control by stress-related treatments and developmental cues, as indicated by the different pattern of regulatory motifs found in each promoter sequence (Fig. 1A). Under stress, an activation of senescence-like program is known to occur (Buchanan-Wollaston and Ainsworth, 2003). During this process, the nitrogenated molecules, such as proteins and nucleic acids, are degraded to release ammonium, amino acids and purine bases, that travel through the phloem to be reassimilated in the growing parts of the plant (Feller and Fischer, 1994). Ureides are synthesized through the oxidative catabolism of purine bases *de novo* generated or released by the degradation of nucleic acids (Zrenner *et al*., 2006).

Therefore, an increment of *de novo* purine synthesis pathway induced by the ammonium released during the protein remobilization may be the source of the ureides accumulated in response to drought stress in the leaves from *P. vulgaris*.

Analysis performed in the leaves of drought-stressed plants of varieties with different tolerance to drought revealed that, neither the expression of *PvPRAT* genes nor the PRAT activity are induced in *P. vulgaris* during situations of water deficit (Fig. 2A and 2B). These results indicate that the increment of ureides observed under drought conditions did not come from the oxidation of purines *de novo* synthesized and, therefore, from the recycling of amino acids released during protein remobilization.

It is known that, as in other organisms, PRATs from plants are sensitive to a feedback regulation by high concentrations of purine nucleotides (Ito *et al*., 1994). In fact, the activity of purified PvPRAT1 is inhibited by AMP *in vitro* (Chapter 4, Table 2). However, PRAT activity is not inhibited in drought-stressed leaves, suggesting that purine nucleotides released during the degradation of nucleic acids do not accumulate but, instead, they are rapidly metabolized to purine bases.

In plants exposed to water deficit, the N_2 fixation is impaired and the nodules became senescent in a degree according to the degree of sensitivity of the genotype (Chapter 2, Fig. 1 and Supplementary Fig. S2). As a consequence, the expression and activity of key enzymes in ureide synthesis such as uricase (UO) (Alamillo *et al*., 2010) and xanthine dehydrogenase (XDH) (Chapter 3, Fig. 3) quickly decreased. Interestingly, the expression of all three *PvPRAT* genes did not change in the nodules of plants subjected to drought stress, not even the *PvPRAT3* (Fig. 2C), which is the predominant isoform expressed in nodules and whose activity is highly related with the N_2 fixation. Further analysis needs to be done to ascertain how is regulated the protein during such conditions in nodules and its possible implication in other metabolic activities which maintain the normal levels of PvPRAT3.

In contrast, the analysis of PvPRAT during seed germination, showed that *de*

novo purine synthesis is activated, through the induction of *PRAT3* expression in at late germination or early seedling development in *P. vulgaris* (Fig. 3), which is consistent with the presence of the Snk-1 motif in its promoter sequence (Fig. 1A). Polayes *et al*. (1984) reported increments in the ureide levels at early stages of seedling development in soybean due to the induction of *de novo* synthesis of purines in the cotyledons. The induction of *PvPRAT3* expression occurred 4 days after imbibition, time in which the highest ureide levels in embryonic axes from *P. vulgaris* are reached (Quiles *et al*., 2009). The data presented here suggest that *PvPRAT3* gene is the one responsible of the increment in *de novo* purine synthesis during germination in *P. vulgaris*. Thus, the induction of *PvPRAT3* isoform probably led to the increase in the ureide concentration observed after the radicle emergence.

During late germination, there should be a high demand of purine nucleotides associated with postgerminative cell division, due to the high requeriments of news DNAs and RNAs to synthesize new proteins (Bewley, 1997; Quiles *et al*., 2009). On the other hand, many seed proteins are remobilized during germination and seedling growth (Shutov *et al*., 2003), releasing amino acids that may act as precursors for *de novo* purine nucleotide synthesis.

The results obtained in this work agree with those described in Chapter 4 about the specifics functions of PvPRATs. Only the expression of *PvPRAT3* gene changed significantly in some of the conditions analyzed in this work such as the treatments with ABA, SNP and ACC, and during germination. In contrast, the expression of *PvPRAT1* and *2* remains unchanged, which indicates that these isoforms appear to be the dedicated to generate the purines required for plant basal development, whereas *PvPRAT3* may be the specialized isoform for the synthesis of purines in situations in which a high nucleotide production is required.

The presence of a large number of light-regulatory motifs in the promoter sequences of *PvPRATs* (Supplementary Fig. S2) suggests a relationship of the genes with light-dependent processes. Hung *et al*. (2004) reported the importance

of *ATase2* gene for chloroplast biogenesis in Arabidopsis, which agree with this hypothesis. Mutants in the PvPRAT homologous, *ATase2* gene, showed light-dependent defects in protein import to the chloroplast. On the other hand, the enhancement of *de novo* purine synthesis at late germination, when seedlings start to become photoautotrophic (Fig. 3) also supports this idea, although further research needs to be done to investigate the implication of *PvPRAT* genes in these processes.

In summary, we show that *PvPRAT* genes are differentially regulated by the treatments and physiological situations analyzed in this work. *PvPRAT3* gene was the only copy showing stress-mediated expression changes, whereas *PvPRAT1* and *PvPRAT2* remained unchanged, supporting the idea of their basal and/or constitutive function. The upregulation of *PvPRAT3* expression during germination but not during drought stress indicated that the ureides synthesized under both physiological situations do not proceed from the same pool of purine nucleotides. Finally, the possible involvement of PRAT in some processes regulated by light is discussed.

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Supplementary material

Supplementary Fig. S1. Identification of putative *cis*-regulatory motifs in the proximal promoter sequences of the *PvPRAT* genes.

Supplementary Fig. S2. Light-responsible *cis*-regulatory motifs found in *PvPRAT*s upstream sequences.

Supplementary Table S1. List of primers used in this study.

PvPRAT1

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-1500  ATATGGACATAAGAGAACATGATCGAACAAAAAAATGTTCATATTGTCAAACACCAAGACACCCTGTTCCAACATTAC
       TATACCTGTATTCTCTTGTACTAGCTTGTTTTTTTACAAGTATAACAGTTTGTGGTTCTGTGGGACAAGGTTGTAATG
            Skn1-motif (Endosperm expression)
-1422  TGGATTATGCATTTCTCGTCATTTATGTTTCATTTCTCACAAACTATTTAACGTACCATTGAAGAAAATATTAAATGA
       ACCTAATACGTAAAGAGCAGTAAATACAAAGTAAAGAGTGTTTGATAAATTGCATGGTAACTTCTTTTATAATTTACT
            CGTCA-motif (MeJa response)              TC-rich repeats (Defense and stress)
-1344  ATGATCATCTTTCATTTATTTTCCTTTTTATGATCAACATATATTTATTTAGTATCGTCTTATATCTCTTATTTTGAT
       TACTAGTAGAAAGTAAATAAAAGGAAAAATACTAGTTGTATATAAATAAATCATAGCAGAATATAGAGAATAAAACTA

-1266  TTTTTTATATCTTTTATTTTTATCTTATTTTGTTTTGCCTTTATTTTATATCTTTTATGTTTTTAGTTATTATTTATT
       AAAAAAATATAGAAATAAAAATAGAATAAAACAAAACGGAAATAAAATATAGAAAATACAAAAATCAATAATAAATAA
       Skn1-motif (Endosperm expression)
-1188  TCTGTCATTCTTTATTTATTTCTGTTTTTTGTTTTTATTTTATTGTTGCTGTTTTTATTTTCTAGACTTATTTATTTT
       AGACAGTAAGAAATAAATAAAGACAAAAAACAAAAATAAAATAACAACGACAAAAATAAAAGATCTGAATAAATAAAA
            GARE-motif (Gibberellin)
-1110  TGCATTTTTTTCTTCTCATTTTTAAGCATTAAGTGTAACATTTGCTTTAGTTATTTTTTAGGATTTTGCATTTAAGGT
       ACGTAAAAAAAGAAGAGTAAAAATTCGTAATTCACATTGTAAACGAAATCAATAAAAAATCCTAAAACGTAAATTCCA
        TCA-element (Salcylic acid)
-1032  AGACACTTCAATATTATTTGTGTGTCCACTATTAATTAATACTAATTGACTTTGATTTTTTTATAATTATGTCCTATA
       TCTGTGAAGTTATAATAAACACACAGGTGATAATTAATTATGATTAACTGAAACTAAAAAAATATTAATACAGGATAT

-954   TTTTTTACTTGAAATTAACATGATTACATAATCAATAATAATAAAAATAAATACTAATTTATGTTTATAGATTCAACT
       AAAAAATGAACTTTAATTGTACTAATGTATTAGTTATTATTATTTTTATTTATGATTAAATACAAATATCTAAGTTGA
                    TC-rich repeats (Defense and stress)
-876   TTTATGTTGTTGTAGAGTTTAATTTTTTATTTTCTTCATTCTTAAAAAAAATAATTCTAAACTAATGTCGGAAGTTGA
       AAATACAACAACATCTCAAATTAAAAAATAAAAGAAGTAAGAATTTTTTTTATTAAGATTTGATTACAGCCTTCAACT

-798   TGCAACCTTTGCTGCTCAAATCCTGTCCTCATTTGCTGAGTTGCTTAATCCAGTTTATGAAGTTCGGACATTTCTCTT
       ACGTTGGAAACGACGAGTTTAGGACAGGAGTAAACGACTCAACGAATTAGGTCAAATACTTCAAGCCTGTAAAGAGAA

-720   AAATAGCGTGTCCGTGTCGGACACTCGTATTGGTGTCGACACTCGTATCGGTGTCGACACTTATCGATGAAATACAAT
       TTTATCGCACAGGCACAGCCTGTGAGCATAACCACAGCTGTGAGCATAGCCACAGCTGTGAATAGCTACTTTATGTTA

-642   TATAACACGGTTCTAACACAATTTTAAAAAAGATAAATACAATAATTTTCTAAAAATTTAAATTTATTGTATAAATTT
       ATATTGTGCCAAGATTGTGTTAAAATTTTTTCTATTTATGTTATTAAAAGATTTTTAAATTTAAATAACATATTTAAA

-564   TTATTATGATTATAAAAATAAGGAACAAATTCTTTTGAACCAATTATTAAAAACATCTTCATATTCTCAAAAAAATCT
       AATAATACTAATATTTTTATTCCTTGTTTAAGAAAACTTGGTTAATAATTTTTGTAGAAGTATAAGAGTTTTTTTAGA

-486   GAAATATATTTGTGCATATAAATATTTATTGTTAATTTATATAATTAATAATTATATAATATATAGATTCATGTACCC
       CTTTATATAAACACGTATATTTATAAATAACAATTAAATATATTAATTATTAATATATTATATATCTAAGTACATGGG

-408   GTATCATATATTTTAGGGATTATACGTATCTCTATATCCGTGTCGTGTCAGTGTCTATATCCATGTTTGTGTTATATA
       CATAGTATATAAAATCCCTAATATGCATAGAGATATAGGCACAGCACAGTCACAGATATAGGTACAAACACAATATAT
            TCA-element (Salicylic acid)
-330   ATTCTCATTTCATCAAGAAAAGGAGGACCAATTCCCAAGGAAGAGTCTCTAAAGTTGTATTGTTCCTACAGTTAAAGC
       TAAGACTAAAGTAGTTCTTTTCCTCCTGGTTAAGGGTTCCTTCTCAGAGATTCAACATAACAAGGATGTCAATTTCG
                                                                    MBS (Drought)
-252   TTAAAGGTATACACAAACGTCTTACTCATTTCCTAATTGATCAAATTTCGTATTTTCCAACAATCATTCCTAATACCC
       AATTTCCATATGTGTTTGCAGAATGAGTAAAGGATTAACTAGTTTAAAGCATAAAAGGTTGTTAGTAAGGATTATGGG

-174   ATGTTTGGTAAAAGAGGCTTTTATCCAGAAAGGGGGTGCAAAAAGAAATTCTTATGGTGCATAAAGTAAAGCCCTTCA
       TACAAACCATTTTCTCCGAAAATAGGTCTTTCCCCCACGTTTTTCTTTAAGAATACCACGTATTTCATTTCGGGAAGT

-96    TCCAATGAAGGAGACTTGTTTTTGGGCCACAAGGGGAAATAACTTCCTTATATGTATATGTACGTCGTATAAAGGAAG
       AGGTTACTTCCTCTGAACAAAAACCCGGTGTTCCCCTTTATTGAAGGAATATACATATACATGCAGCATATTTCCTTC
            →

-16    GGGAAAAAAGGGTGAAGAAGAAAAAAAAGTTAGGTAAACGGAAACAATAACTCCCGAAAGCAAACCTAGAAGAAGCATT
       CCCTTTTTTCCCACTTCTTCTTTTTTTCTTACCATTTGCCTTTGTTATTGAGGGCTTTCGTTTGGATCTTCTTCGTAA

       GGTGTTCCTGAATTTCTTCACTCACTCCAATCTCAGAGTCAGAGTGatg
       CCACAAGGACTTAAAGAAGTGAGTGAGGTTAGAGTCTCAGTCTCACtac
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PvPRAT2

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-1500  AATTATGTAAAGTTTAAAGTTGGTATATAAAATTTTGATAACTAATTAGATATTAATTTATAAACTATTTATTAATAA
       TTAATACATTTCAAATTTCAACCATATATTTTAAAACTATTGATTAATCTATAATTAAATATTTGATAAATAATTATT

-1422  TATAAATTAATTTAAATATTAATTTTTTTCTAAAATAATATATAATTAAATTAATGTAATAATTAATTTTTTTTTAAA
       ATATTTAATTAAATTTATAATTAAAAAAAGATTTTATTATATATTAATTTAATTACATTATTAATTAAAAAAAAAATTT

-1344  TTAGTTTTTATATGATGATTTTCTTATAATAATTACATGTTATAATATAGTAAAACAAATAATATATATATTATTGAATA
       AATCAAAAATATACTACTAAAAGAATATTATTAATGTACAATATTATATCATTTGTTTATTATATATAATAACTTAT

-1266  ATAGCTGAAAAAAACTATTCTAAAAACATAATAAAAAAATTAAATTTTGAATTAAAGCTGTGTATTTTCTTTTATTTA
       TATCGACTTTTTTTGATAAGATTTTTGTATTATTTTTTTAATTTAAAACTTAATTTCGACACATAAAAGAAAATAAAT

-1188  ACAAGAGTGCCTAGAAACCGTTAAAGCTTAAAGGTATACATAAACGTCTTACTCATTTCCTAATTGATAAAATGTCCT
       TGTTCTCACGGATCTTTGGAAATTTCGAATTTCCATATGTATTTGCAGAATGAGTAAAGGATTAACTATTTTACAGGA

-1110  CTTTCGTATTTTCAAACAATAATTCCTAATAGCCATGTTTGCTAACATATGCTTTTATCCAGAAAGGGGGTGCAAAGA
       GAAAGCATAAAAGTTTGTTATTAAGGATTATCGGTACAAACGATTGTATACGAAAATAGGTCTTTCCCCCACGTTTCT

-1032  GAATTTGTTTTGGTGCATAAAGAAAAGCCCTACATCCAATGAAGGAGAAATAACTTTCTATGCTATGTTTGCTTCTAT
       CTTAAACAAAACCACGTATTTCTTTTCGGGATGTAGGTTACTTCCTCTTTATTGAAAGATACGATACAAACGAAGATA

-954   GGTGTACGGTTTACCATAAAAGAATTGAGAAAATATTAATGATTTGGATTCAGATTTTTGCAAGGAAGAGAAGGAGAG
       CCACATGCCAAATGGTATTTTCTT**AACTCTTTTA**TAATTACTAAACCTAAGTCTAAAAACGTTCCTTCTCTTCCTCTC
                             **TC-rich repeats (Defense and stress)**
-876   GATATACCTGTGAACAATAAAT**ATTTTCTCCT**TTCAAAATAAGAGATGTTGAGGAAAAATCATATCACCATATATTAT
       CTATATGGACTCTTGTTATTTATAAAAGAGGAAAGTTTTATTCTCTACAACTCCTTTTTAGTATAGTGGTATATAATA

-798   TTACTTATTTACTCTTATTCATAGGTAAGTATTTTTTAATATTAAAAAATAATATTTTTTATTTATTTATATAAAATG
       AATGAATAAATGAGAATAAGATACCATTCATAAAAAAATTATAATTTTTTATTATAAAAAATAAATAAATATATTTTAC

-720   TGATTTATTTTCTTATTTTATAGTATATATTTAAAATTTTAAAAAATTATTTGAAATTCTCTTTTTTATTTAATTTAT
       ACTAAATAAAAGAATAAAATATCATATATAAATTTTAAAATTTTTTAAT**AAACTTTA**AGAGAAAAAATAAATTAAATA
                                                      **ERE (Ethylene response)**
-642   TGTCGTATTTATATTAATATATTTTTTTACAATTATTATGTTTATGAGTTAGTAATTTATTTGATAATAATTTTTTAT
       ACAGCATAAATATAATTATATAAAAAAATGTTAATAATACAAATACTCAATCATTAAATAAACTATTATTAAAAAATA

-564   GAAATTCTTTTCTTTTGTTAACGTTATATATTTTTTAATTTTTCATTATTATAGAATTTTTAATAATAAAAAATATTA
       **CTTTAAGAAA**AGAAACAATTGCAATATATAAAAAATTAAAAGTAATAATATCTTAAAAATTATTATTTTTTATAAT
       **HSE (Heat stress element)**
-486   TCTTTTATTTATATAAGATCTTATTTATTTTTTAATTTATTTTCTAATTTTATAATACACGTTTAATATTTTAAAAAC
       AGAAAATAAATATATTCTAGAATAAATAAAAAAATTAAATAAAAGATTAAAATATATAGTGCAAATTATAAAATTTTTG
                  **HSE (Heat stress element)**
-408   TTATTTGAAATTTTATTTTATTTAATTTGATGAATACATTTTTTAATTATAATTTTTAAATTAGATCTATTTATATTT
       AAT**AAACT**TTAAAATA**AAAATAAATTAAACTACTTATGTAAAAAATTAATATTAAAAAATTTAATCTAGATAAATATAAA
       **ERE (Ethylene response)**
-330   AACATATATTTATTTTTTAATTTTAATTTTTAAATTTATAAAATATGTGAAAGTTATTTATTTTTAAACAAAAATAAC
       TTGTATATAAATAAAAAATTAAAATTAAAAATTTAAATATTTTATACACTTTCAATAAATAAAAATTTGAAAAATAAG
                  **HSE (Heat stress element)**
-252   TTATTTCATAATTA**AAAAAATTTA**ATAGACAAAGATAAATTTGTAAAATAATATTTAACCATTTAAAAAAAAATTAAAA
       AATAAAGTATTAATTTTTTTAAATTATCTGAAACTATTTAAACATTTTATTATAAATTGGTAAATTTTTTTTAATTTT

-174   CTTTCTCAAAATCATGGTATCACTTAGAATCTCTCATAAATTTTTATCATATATACCCAAATTCAAACATTTTTACTT
       GAAAGAGTTTTAGTACCATAGTGAATCTTAGAGAGTATTTAAAAATAGTATATATGGGTTTAAGTTTGTAAAAATGAA

-96    TCTTT**CAAAT**TTTTTTCCCACTCTTTTCTCCTTTCACTTTCTCGAATCATCCTCCTCGGAACATATCTTATATGCATA
       AGAAAGTTTAAAAAAAGGGTGAGAAAAGAGGAAAGTGAAAGAGCTTAGTAGGAGGAGCCTTGTATAGAATATACGTAT
                   →
-17    TGTAAGG**TATA**AAGGAAGGGAAGAAAAGGGTGAAGAAGAAAAAACTTAGGTAAACGGAATCAATAACTCCCGAAAGCA
       ACATTCCATATTTCCTTCCCTTCTTTTCCCACTTCTTCTTTTTTGAATCCATTTGCCTTAGTTATTGAGGGCTTTCGT

       AACCTAGAAGAAGCATTCGTGTTCCTCAATTTCATCACTCATTTCAATCTCAGACTCCAATCTGAGAGTGAGAGT*Gat*
       TTGGATCTTCTTCGTAAGCACAAGGAGTTAAAGTAGTGAGTAAAGTTAGAGTCTGAGGTTAGACTCTCACTCTCAC*ta*
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PvPRAT3

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                  Skn1-motif (Endosperm expression)
  -1000  ATTTGTGTTGTTGATAAATTGTACAAGCAGCCGCATAATTAGTTCATGCATCCCATACCCATGACCAGTAGAAATTGG
         TAAACACAACAACTATTTAACATGTTCGTCGGCGTATTAATCAAGTACGTAGGGTATGGGTACTGGTCATCTTTAACC
          TCA element (Salicylic acid)                        Skn1-motif (Endosperm expression)
  -922   AAGCCAACTTTTTGTAAATTCTATCTGTGCCTGAATGTCCTCCAAGTAGAATGGAACTCTTCTAAGAACACCAAGACC
         TTCGGTTGAAAAACATTTAAGATAGACACGGACTTACAGGAGGTTCATCTTACCTTGAGAAGATTCTTGTGGTTCTGG
                                                            ABRE (ABA response)
  -844   AGGGAGAAATGAAAGGAATCACTATCTGCCTTTATATAAAAACTCTCTATGTTCCCACGTACACCTAGTACAAGAATT
         TCCCTCTTTACTTTCCTTAGTGATAGACGGAAATATATTTTTGAGAGATACAAGGGTGCATGAGGATCATGTTCTTAA
         Skn1-motif (Endosperm expression)
  -766   GTCATTTTTCTGAATTGCTGAAAAGATTTTAGTTAAGTCTGGATCTGTTTGCACCTCTCATTGCAACTTTTTTGCACC
         CAGTAAAAAGACTTAACGACTTTTCTAAAATCAATTCAGACCTAGACAAACGTGGAGAGTAACGTTGAAAAAACGTGG
                                                        GARE-motif (Gibberellin)
  -688   GAACCAAATTGGTTGAGATAATGCTTGCAGATCCCCTTCCAGCATTCTAGATACTGCATCAGCAGGTCTATTCTCCTT
         CTTGGTTTAACCTTCTCTATTACGAACGTCTAGGGGTTGGTGCTAAGATCTATGACGTACTCGTCCAGATAAGAGGAA
          ARE (Anaerobic induction)                                 TCA element
  -610   TTATGGTTTGTAAATGATGTCAAATTGATATCCCAAAGAACTTTGCAATTCAATCTTGTTGATTCTGTGTAGTCACCT
         AATACCAAACATTTACTACAGTTTAACTATAGGGTTTCTTGAAACGTTAAGTTAGAACAACTAAGACACATCAGTGGA
         (Salicylic acid)                                   Skn1-motif (Endosperm expression)
  -532   GCTGTTCTAGTAAATGTAGCAAGCTTTTCTAATCTGTATAGACTATAAACTTCCTCCTCAACAAATATGGTCATCAAT
         CGACAAGATCATTTACATCGTTCGAAAAGATTAGACATATCTGATATTTGAAGGAGGAGTTGTTTATACCAGTAGTTA

  -454   GCTAAATGAATAATACGAAGGGCATGATTCCCTTCTCATATGTAGATTTTAGCAAGATTAGGAAGAGAAAGCTTTGTT
         CGATTTACTTATTATGCTTCCCGTACTAAGGGAAGAGTATACATCTAAAATCGTTCTAATCCTTCTCTTTCGAAACAA

  -376   AAAATAGTCAATAGGTCCAGACTTTTGGTACTGTTAGAGATATCATTTGATTATGTTAGAAATATGATTTGATTTGAT
         TTTTATCAGTTATCCAGGTCTGAAAACCTAGACAATCTCTATAGTAAACTAATACAATCTTTATACTAAACTAAACTA

  -298   TATATTAAATATGGGAGATATGATATGATTTGATAGCAATATAGATATTTCTCATTTTTTAATATTCATTTTGTTTCT
         ATATAATTTATACCCTCTATACTATACTAAACTATCCTTATATCTATAAAGAGTAAAAAATTATAAGTAAAACAAAGA

  -220   TATTATTGTATCTCTTTTTTCATATAAATAAGAATACTTATGTGTGTTCATAACACATGGAATTCGTTATATTCCTTCT
         ATAATAACATAGAGAAAAAGTATATTTATTCTTATGAATACACACAAGTATTGTGTACCTTAAGCAATATAAGGAAGA

  -142   AGGTTCCTTTCTTATGGTATCAGAGCGGTACCATATTCCATAACCTGAACAAGATGTCAAGTGCCTAACGAAGAAGTA
         TCCAAGGAAAGAATACCATAGTCTCGCCATGGTATAAGGTATTGGACTTGTTCTACAGTTCACGGATTGCTTCTTCAT
                                                    →
  -38    ACTCTAATCTGTGCTTTTCCCTACTTTCTTCTCCTCCTATTCTTGTTTTTCTTCTTATCatg
         TGAGATTAGACACGAAAAGGGATGAAAGAAGAGGAGGATAAGAACAAAAAGAAGAATAGtac
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Supplementary Fig. S1. Identification of putative *cis*-regulatory motifs in the proximal promoter sequences of the *PvPRAT* genes. 1500 bp of *PvPRAT1* and *2* and 1000 bp of *PvPRAT3* up-stream of the transcription start (→) are shown. Shaded grey are the 5´UTR sequences. TATA-box and CAAT-box are underlined. *Cis*-regulatory motifs appear as colour-shaded. Promoter analysis was done using the PlantCARE software (Lescot *et al.* 2002).
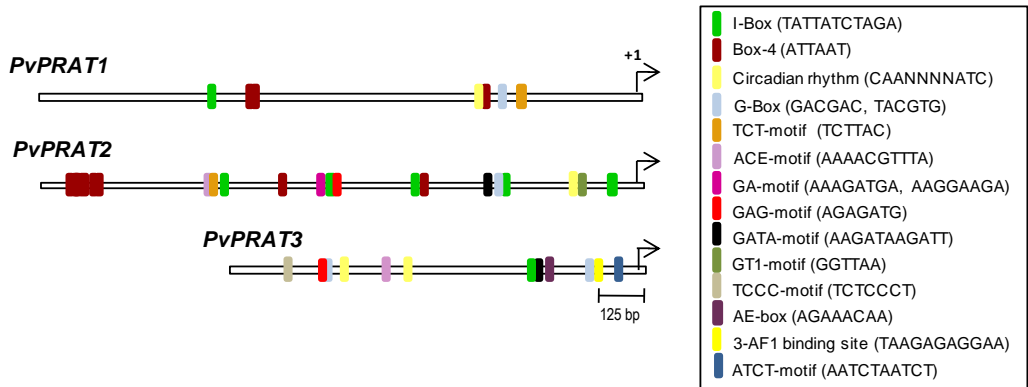
Supplementary Fig. S2. Light-responsible *cis*-regulatory motifs found in PvPRATs upstream sequences.

Supplementary Table S1. List of primers used in this study.

Primer use	Gene	Primers sequences (5´- 3´)
Promoter and 5´-UTR isolation	*PvPRAT1*	GSP1 (TCCTGGCCGCGGTGCTGGAG)
		GSP2 (CATGGGTGTGATTGAAAGGCA)
	PvPRAT2	GSP1 (GTATGCTGCTTTGCCTTTATCT)
		GSP2 (GAAAGTGAGAACATCGGAGAG)
	PvPRAT3	GSP1 (GTTTGGAGGGAGTTGGTGTTGCT)
		GSP2 (CTTCCTGGCCACGGTGCTGGA)
qRT-PCR	*PvAct-2* (KF033666.1)	FWR (GGAGAAGATTTGGCATCACACGTT)
		REV (GTTGGCCTTGGGATTGAGTGGT)
	Pv18S (CV670768.1)	FWR (GACACGGGGAGGTAGTGACAAT)
		REV (ACGAGCTTTTTAACTGCAACAACT)
	PvPRAT1	FWR (GTGAGAGATAATGTCGGTCAAACCATG)
		REV (CATGCATAACCGGCCTTCAGAATCCA)
	PvPRAT2	FWR (TCAAGCGAAAGTGCGGTTTTAGTCAG)
		REV (GTTCCATTCCAAGATGCATCAATCCA)
	PvPRAT3	FWR (GTGGATGTTCGCCATTGGGTTGAG)
		REV (AGCAGGGCGGTTTGAGAATCCAGAG)

CONCLUSIONS

1. The genotypic variation in ureide accumulation under drought stress in *P. vulgaris* is negatively correlated with the degree of N_2 fixation sensitivity of the genotype.

2. Induction of the key enzyme in purine catabolism, PvXDH, indicates that the oxidation of purines is up-regulated for the production of ureides under drought or other stress-related conditions.

3. PvXDH seems to be involved only in the oxidation of purines to increase the concentration of uric acid and, consequently, ureides under stress conditions, but not in the production of ROS.

4. Uric acid, present at high concentration in nodules, protects PvXDH from the inhibition by NO and thus ensuring purine oxidation and ureide production.

5. The three PRAT isoforms are differentially regulated in *P. vulgaris* tissues. The function of PvPRAT1 and 2 is to maintain the housekeeping pool of purines in the plant, whereas PvPRAT3 has evolved to channel the fixed nitrogen to the high purine synthesis needed for ureide production in the nodules.

6. PRAT3 isoform plays an essential role in the determination of the ureidic metabolism.

7. Drought stress does not trigger the induction of the *de novo* synthesis of purines as the expression and activity of PRAT are nor enhanced under this condition.

8. The ureides accumulated in aerial tissues of *P. vulgaris* under drought stress proceed from the oxidation of the purine nucleotides already present in the plant but do not proceed from the oxidation of purines synthesized *de novo*. Therefore, only the nitrogen released by the remobilization of nucleotides and not by the degradation of proteins is used for ureide synthesis.