UNIVERSIDAD DE CÓRDOBA

TESIS DOCTORAL

Effects of symbiotic N fixation on N and water use efficiency in common bean plants: physiological, molecular and biochemical characterization

Efectos de la fijación simbiótica del N sobre la eficiencia en el uso de N y en el uso del agua en plantas de judía: caracterización fisiológica, molecular y bioquímica

MEMORIA PARA OPTAR AL GRADO DE DOCTOR POR LA UNIVERSIDAD DE CÓRDOBA CON MENCIÓN INTERNACIONAL

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Departamento de Botánica Ecología y Fisiología Vegetal

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TITULO: *Effects of symbiotic N fixation on N and water use efficiency in common bean plants: physiological, molecular and biochemical characterization*

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TÍTULO DE LA TESIS:
Efectos de la fijación simbiótica del N sobre la eficiencia en el uso de N y en el uso del agua en plantas de judía: caracterización fisiológica, molecular y bioquímica
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DOCTORANDO/A: Cristina María López Vázquez

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

El trabajo de Tesis Doctoral realizado por Dña. Cristina Mª López Vázquez en el grupo BIO-115 del Departamento de Botánica, Ecología y Fisiología Vegetal ha consistido en el análisis exhaustivo de las respuestas moleculares y fisiológicas de plantas de judía frente al estrés hídrico, demostrando que, la simbiosis con rizobios fijadores de nitrógeno, además de los beneficios medioambientales, tiene efectos muy positivos en la resistencia al estrés hídrico en judía. En este trabajo la doctoranda ha utilizado técnicas moleculares avanzadas, tales como análisis de perfiles transcriptómicos, análisis de expresión por qRT-PCR, clonación, sobreexpresión de proteínas en sistemas heterólogos, edición genómica mediante CRISPR/Cas, análisis metabolómicos, determinaciones analíticas, determinaciones enzimáticas, etc. Con todo ello, la doctoranda no solo ha adquirido los conocimientos técnicos y teóricos para abordar y completar con éxito los objetivos de la tesis, sino que ha obtenido una formación sólida, que garantiza sus solvencia e independencia científica. Es destable que el desarrollo experimental de la presente tesis se ha hecho superando los obstáculos derivados de la pandemia, de las limitaciones en la financiación del grupo, y, aun así, la doctoranda ha abordado y superado con éxito todas las etapas de la Tesis, los experimentos, el análisis de los resultados, la preparación de los manuscritos para su publicación y difusión. Además, Cristina ha realizado una estancia predoctoral altamente exitosa en un centro extranjero, con resultados que se distribuyen en dos de los artículos ya publicados y el capítulo pendiente de publicación, así como el aprendizaje de abordajes nuevos y ha partido al grupo establecer colaboraciones con centros de prestigio internacional. La formación y capacitación de la doctoranda viene refrendada por la presentación de los resultados en varias ponencias en congresos nacionales e internacionales y por las tres publicaciones en revistas del primer cuartil de los que la doctoranda es primera firmante, así como una más como coautora, y probablemente, algunos de los resultados generados por Cristina darán lugar a futuras líneas de investigación en el grupo.

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

Córdoba, 13 de mayo de 2023

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REPORT OF THE IMPACT INDEX AND QUARTILE OF THE JOURNAL CITATION REPORTS (JCR) OF THE PUBLICATIONS DERIVED FROM THE CURRENT THESIS WORK

Research Publication	Impact Factor	JCR Category Rank
López CM; Pineda M; Alamillo JM. (2020). Transcriptomic response to water deficit reveals a crucial role of phosphate acquisition in a drought-tolerant common bean landrace. Plants **9: 445**. https://doi.org/10.3390/plants9040445	3.935	Q1/Plant Science
López CM; Pineda M; Alamillo JM. (2020). Differential Regulation of Drought Responses in Two *Phaseolus vulgaris* Genotypes. Plants **9: 1815.** https://doi.org/10.3390/plants9121815	3.935	Q1/Plant Science
López CM; Alseekh S; Torralbo F; Martínez Riva FJ; Fernie AR; Amil-Ruiz F; Alamillo JM (2023). Transcriptomic and metabolomic analysis reveals that symbiotic nitrogen fixation enhances drought resistance in common bean. Journal of Experimental Botany **erad083.** https://doi.org/10.1093/jxb/erad083	7.378	D1/Plant Science
Alamillo JM, **López CM,** Martínez Rivas FJ, Torralbo F, Bulut M and Alseekh S. (2023) Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein and hairy roots: a perfect match for gene functional analysis and crop improvement. Current Opinion in Biotechnology 79.	10.279	D1/ Biotechnology & applied microbiology D1/ Biochemical research methods

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Book Publication	Editorial	ISBN
López CM, and Alamillo JM. (2021) Fijación Biológica de Nitrógeno. Colección CID. Serie Biociencias. Ed. Aula Magna, McGraw-Hill. UCO-Press.	Aula Magna, McGraw-Hill. UCO-Press.	978-84-18392900

REPORT OF THE PRESENTATIONS TO CONGRESSES DERIVED FROM THE CURRENT THESIS WORK

Poster Communication:

1. Josefa Muñoz Alamillo**; Cristina María López Vázquez**; Manuel Pineda Priego

RNA-seq analysis revealed key genes associated with drought stress response in a tolerant phaseolus vulgaris genotype

XXIII Reunión bianual de la Sociedad Española de Fisiología Vegetal / XVI Congreso Hispano-Luso de Fisiología Vegetal. Pamplona (España). Junio 2019.

2. Josefa Muñoz Alamillo; **Cristina María López Vázquez**; Manuel Pineda Priego

Molecular analysis of the recycling of purine nucleotides in common bean

XXIII Spanish Society of Plant Biology / XVI Spanish Portuguese Congress of Plant Biology. Pamplona (España). June 2019.

3. Josefa Muñoz Alamillo; **Cristina María López Vázquez**

Molecular and functional characterization of common bean adenine phosphoribosyltransferase

XV National Meeting of Nitrogen Metabolism. Córdoba (Spain). February 2022.

Oral Communication:

1. Josefa Muñoz Alamillo**; Cristina María López Vázquez**; Manuel Pineda Priego

El análisis transcriptómico reveló genes clave asociados con la respuesta al estrés hídrico en un genotipo tolerante de Phaseolus vulgaris

VIII Scientific Congress of Researchers in Training of the University of Córdoba. Cordoba (Spain). February 2020.

2. Josefa Muñoz Alamillo; **Cristina María López Vázquez**; Manuel Pineda Priego

Uso de herramientas de edición génica para esclarecer la función de proteínas codificadas por familias multigénicas en judía

IX Scientific Congress of Researchers in Training of the University of Córdoba. Cordoba (Spain). May 2021.

3. **Cristina María López Vázquez** and Josefa Muñoz Alamillo

Molecular and biochemical characterization of adenine phosphoribosyl transferase from Phaseolus vulgaris

2nd PhD Meeting in Plant Science organized by PhD students such as a satellite activity of the XXV Meeting of the Spanish Society of Plant Biology / XVIII Spanish Portuguese Congress of Plant Biology. Vigo (Spain). July 2021

4. **Cristina María López Vázquez** and Josefa Muñoz Alamillo

Molecular and biochemical characterization of adenine phosphoribosyl transferase from Phaseolus vulgaris

XXIV Meeting of the Spanish Society of Plant Biology XVII Spanish Portuguese Congress on Plant Biology. Vigo (Spain). July 2021.

5. **Cristina María López Vázquez** and Josefa Muñoz Alamillo

Nitrogen fixation ameliorates the negative drought effects in a commercial common bean cultivar

XV National Meeting of Nitrogen Metabolism. Córdoba (Spain). February 2022.

6. **Cristina María López Vázquez** and Josefa Muñoz Alamillo

La fijación de N_2 mejora los efectos negativos de la sequía en un cultivo commercial de frijol común

X Scientific Congress of Researchers in Training of the University of Córdoba. Córdoba (Spain). May 2022.

7. Josefa Muñoz Alamillo and **Cristina María López Vázquez**

CRISPR-Cas9 targeted knockout of common bean adenine phosphoribosyltransferases reveals the functional divergence of this highly uncharacterized gene family in plants

44th National Congress of the Spanish Society of Biochemistry and Molecular Biology. Málaga (Spain). September 2022.

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ABBREVIATIONS

AAH: Allantoate deaminase

ABA: Abscisic acid

ALC: Allantoic acid

ALN: Allantoin

AMP: Adenosine monophosphate

APRT: Adenine phosphoribosyltransferase

ASN: Asparagine

BNF: Biological nitrogen fixation

C: Carbon

Cer: Sphingolipids

CG/MS: Gas chromatography coupled with mass spectrometry

CKs: Cytokinin

DEGs: Differential expression genes

DF: Drought N_2-fixation

DHA: 2,8-dihydroxyadenine

DN: Drought NO_3^--fertilized

DNA: Deoxyribonucleic acid

DW: Dry weight

EDTA: Ethylenediaminetetraacetic acid

FAOSTAT: Food and Agriculture Organization Corporate Statistical Database

FDR: False discovery rate

FPKM: Kilobase per million mapped reads

FW: Fresh weight

GLN: Glutamine

GMP: Guanosine monophosphate

GO: Gene ontology

H_2O_2: Hydrogen peroxide

HGPRT: Hypoxanthine/guanine phosphoribosyltransferase

HIU: 5-Hydroxyisourate

IAA: 3-indoleacetic acid

ICA: Indole-3-carboxylic acid

IMP: Inosine monophosphate

IPCC: Intergovernmental Panel on Climate Change

JA: Jasmonic acid

LC/MS: Liquid chromatography coupled with mass spectrometry

MSTFA: N-Methyl-N-trimethylsilyltrifluoroacetamide

N: Nitrogen

N_2: Atmosferic nitrogen

N_2O: Nitrous oxide

NH_4^+: Ammonium

NO_3^-: Nitrate

$OH^{\cdot}$: Hydroxyl radicals

OHCU: 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline

P: Phosphate

PC: Phosphatidylcholine

PCA: Principal component analysis

PE: Phosphatidylethanolamine

PP2C: Phosphatase 2C

PPFD: Photosynthetic photon flux density

PRA: Phosphoribosylamine

PRAT: Phosphoribosyl pyrophosphate amidotransferase

PRPP: Phosphoribosylpyrophosphate

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RT: Room temperature

RWC: Relative water capacity

SA: Salicylic acid

SOD: Superoxide dismutase

SW: water-saturated weight

SWC: Soil water capacity

TAG: Triacylglycerides

TFs: Transcription factors

XDH: Xanthine dehydrogenase

RESUMEN

La judía común (*Phaseolus vulgaris*) es uno de los cultivos con mayor importancia, no solo por su alto valor nutricional, sino también medioambiental, ya que, como leguminosa, la judía común tiene la capacidad de fijar el N atmosférico (N_2) en sus nódulos radiculares, disminuyendo así el uso de fertilizantes y su consecuente emisión de gases de efecto invernadero. Sin embargo, la fijación de N_2 es particularmente sensible a diferentes condiciones adversas como la sequía, cuyos efectos se están agravando por el cambio climático. Por tanto, estudiar los efectos de la sequía en los cultivos de estas leguminosas es de especial relevancia en regiones áridas como Andalucía (España), donde la disponibilidad de agua es cada vez más escasa y siendo este uno de los factores que más limitan su productividad.

El presente trabajo de tesis está organizado en 4 capítulos en los cuales se estudia la regulación a nivel molecular, bioquímico y fisiológico de los mecanismos de defensa de las plantas de *P. vulgaris* frente al estrés hídrico. Recientemente, el desarrollo de tecnologías -ómicas, que permiten evaluar cambios moleculares a gran escala, se ha convertido en una herramienta de gran utilidad para la evaluación de la respuesta al estrés en plantas. Por tanto, en el capítulo 1 se analizaron los cambios transcriptómicos (RNA-seq) en respuesta al estrés hídrico en una variedad local de *P. vulgaris* (PHA-0683) resistente a la sequía. Para ello se utilizó tejido foliar de plantas control y plantas sometidas a 10 días de sequía, cultivadas en condiciones de fijación de N_2. El análisis mostró la disminución de la expresión de genes asociados con la inhibición de la respuesta del ácido abscísico (ABA), la principal hormona de respuesta al estrés en la planta. Además, la prevalencia los genes expresados diferencialmente (DEG) relacionados con la respuesta a la carencia de fosfato (P) y el análisis fisiológico de plantas PHA-0683 en respuesta a sequía con diferentes concentraciones de P, demostraron que, entre los mecanismos de tolerancia de esta variedad está, precisamente, la capacidad de movilizar fosfato en condiciones de estrés.

La domesticación, la expansión global y las prácticas agrícolas locales han implicado el desarrollo de una amplia variedad de líneas o genotipos de judía común con diferentes grados de resistencia al estrés hídrico. Por lo que en el capítulo 2 se llevó a cabo la comparación de la respuesta molecular y fisiológica al estrés hídrico de dos variedades de judía. Para ello se utilizaron la variedad local de *P. vulgaris* (PHA-0683), con un alto

grado de tolerancia a los periodos de sequía, y una línea comercial (PMB-0220) de tolerancia intermedia a la sequía, pero mayor productividad. La comparación mostró que mientras la variedad local, PHA-0683, mantenía relativamente estables sus parámetros fisiológicos en respuesta a diez días de falta de riego, la variedad PMB-0220 disminuía su contenido hídrico foliar, su biomasa aérea y el contenido en clorofilas en las hojas más viejas. El análisis de los niveles de expresión de genes relacionados con la respuesta al ABA y de varios factores de transcripción que regulan la senescencia en la planta, demostró que había claras diferencias cualitativas y cuantitativas en los patrones de expresión de varios de estos reguladores en respuesta a la sequía entre las dos variedades.

Con el fin de incrementar la tolerancia a la sequía en cultivos de judía más productivos, en el capítulo 3 se analizó de forma más completa la respuesta al estrés hídrico en plantas de la línea comercial PMB-0220 que es menos tolerante a la sequía que PHA-0683, pero tiene mayor productividad. A pesar de las ventajas que supone cultivar leguminosas como la judía común en condiciones de fijación de N_2, muchos agricultores prefieren el uso de fertilizantes nitrogenados que aseguren una buena cosecha. En este capítulo se estudió la respuesta a la sequía en plantas de judía PMB-02202 cultivadas con nitrógeno inorgánico procedente de la fertilización, o en condiciones de simbiosis y fijación de nitrógeno. Para ello, en este capítulo se combinaron análisis transcriptómicos y metabolómicos con los análisis fisiológicos. El estudio de la expresión diferencial de genes mostró que las plantas cultivadas con fijación de N_2 tenían mayor expresión de genes asociados con la síntesis de ABA y de respuesta al estrés hídrico, incluso en ausencia de estrés, comparadas con las plantas fertilizadas con NO_3^-. Así mismo, también se observó la inducción de genes relacionados con el metabolismo de los azúcares solubles y el mantenimiento del balance osmótico en plantas fijadoras de N_2 y sometidas a sequía. El análisis metabolómico confirmó que las plantas en simbiosis y sometidas a 10 días de sequía también acumulaban mayor concentración de metabolitos de defensa a la sequía, como la propia hormona ABA y ureidos (alantoína), azúcares solubles, y otros metabolitos que mantienen el balance osmótico en la planta como rafinosa y prolina, además de diversos lípidos que ayudan en el mantenimiento de la fluidez de las membranas. Por último, el estudio fisiológico confirmó que las plantas fijadoras de N_2 mantenían mejor el contenido hídrico foliar y estaban mejor adaptadas que las plantas fertilizadas con NO_3^-. En este capítulo 3, también se realizó un experimento de recuperación tras el tratamiento de estrés hídrico, en el que las plantas volvieron a regarse después de los 10 días de sequía y se

cultivaron hasta el momento de la recogida de frutos. Tras la rehidratación, las plantas en fijación de N_2 recuperaron el intercambio de gases, tasa de fotosíntesis y la producción de frutos, mientras que las plantas fertilizadas con NO_3^- no consiguieron recuperarse de la sequía, mantuvieron valores reducidos de intercambio de gases y fotosíntesis a pesar de la rehidratación y disminuyeron la productividad de su cosecha.

Varios estudios han indicado el papel positivo de los ureidos en diferentes situaciones de estrés. Los nucleótidos de purina, precursores de los ureidos, pueden sintetizarse mediante la síntesis *de novo* u obtenerse mediante vías de reciclaje de las bases de purina. Dos enzimas son las responsables del reciclaje de purinas, la adenina fosforribosiltransferasa (APRT) que cataliza el reciclaje de adenina y la hipoxantina guanina fosforribosiltransferasa (HGPRT) que cataliza el reciclaje de guanina e hipoxantina. En el capítulo 4 se aborda la caracterización molecular, bioquímica y funcional de la primera de estas dos enzimas. La adenina fosforribosiltransferasa está codificada por cuatro genes en *P. vulgaris*. El análisis filogenético de las secuencias, así como también la sobreexpresión de las cuatro proteínas fusionadas a -YFP (Yellow fluorescent protein) en plantas de *Nicotiana benthamiana*, reveló que las APRTs de judía se agrupaban en dos "clusters", dos de las cuatro proteínas tienen localización cloroplástica, mientras que las otras dos se localizan en el citosol. La caracterización bioquímica, el patrón de expresión génica y los niveles de actividad en los diferentes tejidos de *P. vulgaris* mostraron que PvAPRT1 y PvAPRT5 eran las proteínas predominantes y que, curiosamente, tenían altos niveles de expresión en raíz y nódulos. Con el fin de dilucidar el papel específico que llevan a cabo estas isoformas, se generaron raíces mutadas en cada uno de los dos genes mediante edición génica usando la tecnología CRISPR/Cas9. Finalmente, el análisis fisiológico y metabolómico de estas raíces transgénicas reveló que, si bien el gen PvARPT1 actúa principalmente en la vía de reciclaje de la adenina, el gen PvAPRT5 participa en la regulación de los niveles de citoquininas, modificando el crecimiento de la raíz y los nódulos de la planta. Además, tanto los datos transcriptómicos de los capítulos anteriores, como los perfiles metabolómicos de las raíces con mutaciones en los genes que codifican APRTs indican que estas enzimas tienen también un papel importante en las respuestas a estrés en las plantas de judía.

SUMMARY

Common bean (*Phaseolus vulgaris L*) is one of the most important crops, not only for its high nutritional value, but also for its environmental advantages. This is because, as a legume, common bean can fix atmospheric N (N_2) in its root nodules, thus reducing the requirement of fertilizers and its consequent emission of greenhouse gases. However, N_2 fixation is particularly sensitive to various adverse conditions such as drought, whose effects are being aggravated by climate change. For this reason, farmers opt for the use of fertilizers that ensure a good harvest. Therefore, studying the effects of drought on these legumes is of special relevance in arid regions such as Andalusia (Spain), where the availability of water is increasingly scarce, and drought is one of the factors that most limit crop productivity.

This Thesis work is organized into 4 chapters in which the regulation at the molecular, biochemical, and physiological level of the response mechanisms of *P. vulgaris* plants against water stress is studied. Recently, transcriptomics has become a very useful tool for the evaluation of the environmental responses in different plant species. Therefore, in chapter 1 we analysed the transcriptomic changes (RNA-seq) in response to water stress in a *P. vulgaris* (PHA-0683) landrace resistant to drought. Leaves from control plants and plants subjected to 10 days of drought, cultivated under N_2 fixation conditions, were used. The transcriptomic analysis showed the decrease in the expression of genes associated with the inhibition of the abscisic acid (ABA) response, the main stress response hormone in the plant. In addition, they were a significant number of differentially expressed genes (DEG) related to the response to phosphate (P) deficiency. Relevance of P acquisition under stress was confirmed by drought experiments performed with various concentrations of P, thus revealing that, among the tolerance mechanisms of this resistant landrace is the ability to mobilize phosphate under stress conditions.

Domestication, global expansion, and local agricultural practices have involved the development of a wide variety of common bean lines or genotypes with varying degrees of resistance to water stress. Therefore, in chapter 2, the study of the molecular and physiological response to water stress of two common bean genotypes was carried out. For this, the *P. vulgaris* landrace (PHA-0683), previously described as having a high degree of tolerance to drought, and the market class breeding line Great Northern (PMB-0220), with intermediate tolerance to drought, but higher productivity, were compared.

The comparison showed that while the landrace PHA-068 kept its physiological parameters stable, the market class PMB-0220 decreased its leaf water content, its shoots biomass, and the chlorophyll content in its oldest leaf tissue. The analysis of expression levels of relevant genes related to the ABA response and transcription factors that regulate senescence showed clear qualitative and quantitative differences in the expression patterns in response to drought between the two genotypes.

To increase tolerance to drought in more productive bean crops, in chapter 3 the response to water stress was analyzed in greater depth in plants of the commercial line PMB-0220, which despite being less tolerant to drought than PHA-0683, has higher productivity. Despite the advantages of growing legumes such as N_2 fixation common beans, many farmers prefer the use of nitrogen fertilizers to ensure a good harvest. In this chapter, the response to drought was studied in PMB-0220 bean plants grown with inorganic nitrogen from fertilization, or under symbiosis and nitrogen fixation conditions. To do this, in this chapter we combined transcriptomic and metabolomic analysis, together with physiological analysis. Leaf tissue from plants subjected to 10 days of drought and fertilized with nitrate or in symbiosis with rhizobia was used in the -omic analyses. The study of differential gene expression showed that plants grown with N_2 fixation had higher induction of genes associated with ABA synthesis and response to water stress, even in the absence of stress, compared to plants fertilized with NO_3^-. Likewise, the induction of genes related to the metabolism of soluble sugars and with the maintenance of osmotic balance in N_2-fixing plants subjected to drought was also observed. The metabolomic analysis confirmed that plants in symbiosis and subjected to 10 days of drought accumulated a higher concentration of drought response metabolites, such as the ABA hormone itself, ureides (allantoin), soluble sugars, and other metabolites that maintain the osmotic balance in the plant, as raffinose and proline, as well as various lipids that help maintain membrane fluidity. The physiological study confirmed that N_2-fixing plants maintained their leaf water content better and were better adapted than plants fertilized with NO_3^-. In this chapter, a recovery experiment was carried out after the water stress treatment, in which the plants were irrigated again after 10 days of drought and cultivated until the pod collection. The N_2-fixing plants, after rehydration, recovered their gas exchange values, their photosynthesis rate and even maintained pod production, whereas the NO_3^- fertilized plants did not manage to recover from the drought, they

maintained reduced gas exchange and photosynthesis values despite rehydration and decreased their pod and seeds yields.

Several studies have indicated the positive role of ureides in different stress situations. Purine nucleotides, precursors to ureides, can be synthesized by the *de novo* synthesis or obtained by salvage pathways of purine bases. Two enzymes are responsible for purine salvage, adenine phosphoribosyltransferase (APRT) which catalyzes the adenine salvage and hypoxanthine guanine phosphoribosyltransferase (HGPRT) which catalyzes the salvage of guanine and hypoxanthine. In chapter 4, the molecular, biochemical, and functional characterization of the first of these two enzymes is addressed. Adenine phosphoribosyltransferase is encoded by four genes in *P. vulgaris*. Phylogenetic analysis, as well as the overexpression of the four coded proteins fused to -YFP (Yellow fluorescent protein) in *Nicotiana benthamiana* plants, revealed that the common bean APRTs were grouped into two "clusters", two of the four proteins have possible location to chloroplast, while the other two are in the cytosol. The biochemical characterization, gene expression pattern, and the levels of activity in the different tissues of *P. vulgaris* showed that PvAPRT1 and PvAPRT5 were the predominant proteins and that, interestingly, they had high levels of expression in roots and nodules. To elucidate the specific role played by these isoforms, roots mutated in each of the two genes were generated by gene editing using CRISPR/Cas9 technology. Finally, the physiological and metabolomic analysis of these transgenic roots revealed that, although the PvAPRT1 is mainly involved in the adenine salvage pathway, the PvAPRT5 could function in the regulation of cytokinin levels, modifying root growth and nodulation. In addition, both the transcriptomic data from the previous chapters, and the results of the metabolomic profiles of roots with mutations in the genes that encode APRTs, indicate that these enzymes also have an important role in stress responses in common bean plants.

GENERAL INTRODUCTION

Climate change and legume crops

For the future year 2050, a considerable increase in the world population is expected, reaching up to 9 billion people (Gerland *et al.*, 2014). In response to increased demand for food that comes with this larger population, it will be necessary to extend the crops surface and to meet the food needs. However, the larger cultivation surface, the greater consumption of nitrogenous fertilizers for its growth will be required (Smil, 1997; Elliott *et al.*, 2014). Part of the nitrogen (N) used by these fertilizers is lost through leaching or volatilization in form of greenhouse gases such as nitrous oxide (N_2O) (Vergé *et al.*, 2007). This aggravates the consequences of a current problem, climate change.

Currently, the most notable effect of climate change is the global warming, which leads to a decrease in crop yields (Arora, 2019*a*). According to the Intergovernmental Panel on Climate Change (IPCC), since the mid-twentieth century, human influence is the main source of global warming, and is leading to higher annual net costs for crop production. The increase in cultivated area and global warming will further limit the availability of water, which is one of the factors that influences agricultural productivity (Elliott *et al.*, 2014; Noya *et al.*, 2018). Droughts, which are exacerbated by global warming, can make it difficult for farmers to work and put food security at risk (Ziska *et al.*, 2016). To meet these challenges, it is necessary to establish farming practices which would be more sustainable. In this aspect, legumes crops could play an important role.

The legume family (*Fabaceae*), made up of ~ 670 genera and ~18.000 species, is second only to the *Gramineae* family in importance to humans (Polhill *et al.*, 1981). Legumes represent 27% of the primary crops production used as forage or grain (Graham and Vance, 2003). Some of the most economically important grain legumes are chickpeas (*Cicer arietinum*), lentils (*Lens culinaris*), alfalfa (*Medicago sativa*), soybeans (*Glycine max*) and common beans (*Phaseolus vulgaris*). Of these, soybean crop is the one with the highest production, followed by common bean, according to the statistical data provided by the FAOSTAT in 2020 (Figure 1). These legumes provide a great source of protein in the human diet (Tharanathan and Mahadevamma, 2003). In addition, legume seeds can help to prevent diseases such as hypercholesterolemia and hypertension (Arnoldi *et al.*, 2015). Moreover, with the objective to reach a more sustainable agriculture, legumes have the advantage of being able to establish symbiotic associations with diazotrophic soil

rhizobia able to fix and use atmospheric nitrogen (N_2), thus reducing the needs for nitrogenous fertilizers (Brockwell *et al.*, 1995; Sprent, 2002; Lemke *et al.*, 2007).

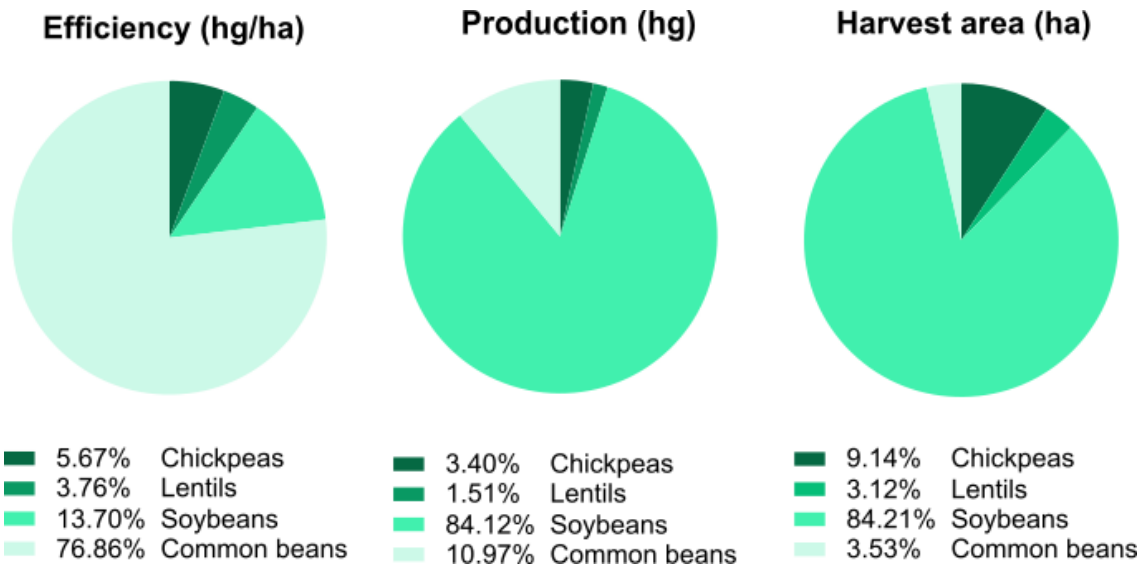

Figure 1. Global legume cultivation according to FAOSTAT. Data on efficiency, production, and cultivation area of the greater economic importance legume crops (chickpeas, lentils, soybeans, common beans) (https://www.fao.org/faostat/es/#data/QCL).

Biological fixation of atmospheric nitrogen

Nitrogen (N) is one of the most abundant chemical elements in living matter, present in amino acids, nucleic acids, and many other biomolecules. Therefore, together with water, N is the most limiting factor in plant growth and production.

In general, plants use the N present in the soil in form of nitrate (NO_3^-) or ammonium (NH_4^+). Nevertheless, in conditions of limiting soil N, legumes have the ability to take prokaryotic fixed atmospheric N_2 (Sprent, 2002). By themselves, eukaryotic plant cells cannot use molecular N_2. But, by the symbiotic association with diazotrophic bacteria of the *Rhizobiaceae* family (α- and β-proteobacteria), legumes can use the N fixed by the bacteria in the process called biological N_2 fixation (BNF) (Sawada *et al.*, 2003). Among the legume crop species that can associate with rhizobia and take advantage of the BNF are some of the most important grain legumes, as soybeans, peanuts, peas, common beans, lentils, broad beans, chickpeas and lupine (Andrews and Andrews, 2017).

From the symbiotic association between plant and rhizobium, specialized organs called nodules are formed. These nodules house the bacteroides that receive nutrients, such as dicarboxylic acids, from the plant, while the plant obtains the fixed N_2 in the form of NH_4^+ (Udvardi and Day, 1997). The formation of NH_4^+ comes from the reduction of N_2

by the prokaryotic nitrogenase complex in the nodules (Postgate, 1982). This process requires a high energy cost, that can also be increased if there is a limiting flow of electrons (Simpson and Burris, 1984; Burgess and Lowe, 1996). However, despite the energy cost, the BNF gives N_2-fixing legumes the advantage of growing in N-poor soils. In addition, plants cultivated under N_2 fixation allow to generate a 30% supply of N to the soil for the next agricultural growing season (Andrews *et al.*, 2007, 2013). Therefore, it is possible to increase the fertility of the soil to carry out sustainable agricultural practices (Hossain *et al.*, 1996).

Despite all the advantages of BNF, many farmers are reluctant to limit the use of nitrogen fertilizers, to ensure a good harvest in their crops. This is due to the fact that the symbiosis with rhizobia is particularly sensitive to adverse conditions, such as drought, thus limiting their use (Araújo *et al.*, 2015).

Common bean cultivation

After soybeans, common bean (*Phaseolus vulgaris L.*) is the most widely cultivated and important grain legumes for human consumption (Broughton *et al.*, 2003; Beebe, 2012). Originally from America, common bean has been domesticated in at least two domestication centers, the Andean and Mesoamerican (Gepts and Bliss, 1986; Myers and Kmiecik, 2017). After the discovery of America, the cultivation of common bean spread to Europe and Africa (Gepts and Bliss, 1988). The different races of common bean are classified depending on their biochemical and morphological characteristics. Specifically, common beans from the Mesoamerican area are classified into 3 races (Mesoamerica, Durango and Jalisco), and same for the Andean common beans (New Granada, Chile and Peru) (Singh *et al.*, 1991).

Surprisingly, although BNF is rapidly inhibited by drought conditions (King and Purcell, 2005), legumes are among the first plant species capable of colonizing and growing in arid and nutrient-poor soils. There are cultivated common beans varieties that present varying degrees of tolerance or sensitivity to adverse conditions (Riveiro, 2012; Coleto et al., 2014) (Figure 2). According to several studies, among the different races from the Mesoamerican heritage, the one with the highest resistance to water stress is the Durango race. In contrast, breeds from the Andean heritage, which have been developed in humid and temperate areas, evolved less adaptations to abiotic stress (Rosales-Serna et al., 2004; Muñoz-Perea et al., 2006). The most common commercial varieties of the Durango breed

are Bayo, Flor de Mayo, Ojo de cabra (located in the Mexican Altiplano); Rosa, Pinta, Roja (North America) and Great Northern (America, Europe, and Asia) (Singh et al., 1991b; Beebe et al., 2000). Within the Andean heritage, Nueva Granada is the one with the largest seed size and productivity. Among the most common commercial lines are Manteca, Radical, Alubia roja oscura y Alubia roja clara, Cranberry and so on.

Figure 2. Common bean varieties used in this work. (a) Flower and seeds of *P. vulgaris* landrace PHA-0683. (b) Flower and seeds of the commercial line of *P. vulgaris* "Matterhorn" Great Northern PMB-0220.

This wide variety of common bean genotypes with different degrees of tolerance to water stress and productivity has been obtained by domestication processes and local farming practices (Frahm *et al.*, 2004; Beebe *et al.*, 2013). Nowadays, scientists consider that genetic improvement and identification of molecular markers are key in improving crop yields and increasing their tolerance to adverse conditions such as drought.

Amide and ureide legumes

Depending on how the nitrogenous compounds originated from the fixation of N_2 are stored and transported to plant tissues, legumes are classified into two types. Legumes that transport nitrogenous compounds in the form of asparagine (ASN) and glutamine (GLN) are known as amidic, while those that transport nodule fixed N in form of ureides, allantoin (ALN) and allantoate or allantoic acid (ALC), are known as ureidic legumes (Atkins, 1991).

Amide legumes, such as peas, alfalfa and lotus are crops from temperate climates. The asparagine used by these legumes comes from nodule nitrogen fixation and from Krebs cycle intermediates, such as oxoglutarate and malate. In addition, amide legumes use these molecules as organic N-transport, regardless of whether the N comes from BNF or from fertilization with NO_3^- or NH_4^+. Interestingly, except for the *Lotus* genus that does have determinate nodules, amidic legumes usually bear indeterminate nodules (Tajima *et al.*, 2000). Indeterminate nodules have an elongated shape and maintain meristematic cells at their distal end that are active throughout the life of the nodule (Hirsch, 2012).

On the other hand, ureidic legumes such as common beans and soybeans, typical of tropical climates, use the ureides ALN and ALC only when the N comes from the BNF. By contrary, when cultivated with a mineral source of nitrogen, ureidic legumes use also asparagine and glutamine to export nitrogen to the rest of the plant, behaving as the amide type legumes and the rest of the plants (McClure and Israel, 1979; Pate *et al.*, 1980; Schubert, 1986). Although synthesis of ureides requires more enzymatic steps than amides synthesis, energy expenditure is no greater than that for amide synthesis. In addition, transport of ureides is a carbohydrate saving strategy, since synthesis of asparagine and glutamine uses 2 and 2.5 molecules of C for each N incorporated, respectively. In contrast, the synthesis of ureides requires only one molecule of C per molecule of N (Atkins, C., & Smith, 2000). Unlike amidic legumes, ureidic legumes have determinate nodules that have a spherical shape and a transient meristematic activity, thus they have a determinate growth and lifespan (Hirsch, 2012).

Ureides in stress situations

Measurement of ureides has been used to estimate the rate of symbiotic fixation in ureidic legumes since it was assumed that high accumulation of ureides only occurred to accumulate de fixed N_2 in ureidic plants. Nevertheless, different environmental factors and the state of plant development can influence the concentration of ureides, regardless of the BNF. For instance, plants in the reproductive or senescent phase have higher concentrations of ureides (Schubert, 1981; Díaz-Leal *et al.*, 2012). What's more, some non-legume plants are also known to accumulate high concentrations of ureides (Schmidt and Stewart, 1998).

Abiotic stress can also influence ureide concentrations and, it was shown that soybeans genotypes sensitive to drought conditions accumulate more ureides than tolerant ones

(King and Purcell, 2005). So, for a long time, these metabolites have been associated with greater sensitivity to drought. One of the earlier processes affected by drought in plant is BNF and, it has been observed that BNF in ureidic legumes is even more sensitive than in amide legumes (Sinclair and Serraj, 1995*a*; Serraj *et al.*, 1999*a*). Therefore, different studies proposed that the ureides accumulated during water stress were associated with the inhibition of BNF (Serraj *et al.*, 1999*c*; Purcell *et al.*, 2000, 2004; Ladrera *et al.*, 2007).

Contrary to their possible negative role for BNF, previous studies in our group showed accumulation of ureides in root, stem, and leaf tissues, but not in nodules of common bean plants under drought stress (Alamillo *et al.*, 2010; Coleto *et al.*, 2014). Furthermore, this accumulation of ureides occurred long after BNF had already been inhibited by drought. These results indicated that ureides cannot be the cause of the inhibition of this process. In addition, it indicates that the increase in ureide levels should came from vegetative tissues, probably due to purine nucleotides degradation and the induction of xanthine dehydrogenase (XDH) and allantoinase activities (Coleto et al., 2014; 2019).

The possible relationship of abscisic acid (ABA) with the accumulation of ureides has also been pointed out (Alamillo *et al.*, 2010). ABA is the main phytohormone that promote the response osmotic and drought stress. Specifically, ABA influences the regulation of different physiological processes, including stomatal conductance and photosynthesis inhibition, which have a great importance for the tolerance to drought (Farquhar and Sharkey, 1982; Downton *et al.*, 1988). In addition, it has been shown that *Arabidopsis* mutants with a high accumulation of the ureide allantoin promote the activation of the ABA accumulation pathways (Watanabe *et al.*, 2014*b*).

On the other hand, there are studies that link ureides with protection against reactive oxygen species (ROS) caused by different types of stress (Nakagawa *et al.*, 2007*b*; Brychkova *et al.*, 2008). *Arabidopsis* plants with suppressed XDH activity, a key enzyme in ureide synthesis, were more sensitive to drought stress and accumulated more ROS. Interestingly, the drought hypersensitivity of these plants was reversed when exogenous uric acid, which is the product generated by the XDH activity and the precursor of the ureides, was applied (Watanabe *et al.*, 2010, 2014*a*).

In summary, allantoin and allantoate are the main nitrogen remobilizing molecules in tropical legumes under N_2-fixation (Schubert, 1986). But according to the new studies,

ureides could play a key role influencing signaling pathways and protection against different types of stress, such as drought (Alamillo *et al.*, 2010; Watanabe *et al.*, 2014*b*).

Purine nucleotide metabolism

Ureide synthesis comes from the degradation of purine nucleotides (Tracey, 1955). Apart from being the precursor of ureides, purine nucleotides are basic components of nucleic acids (DNA and RNA), energy metabolism and act as substrates for the synthesis of nicotinamide adenine dinucleotides (NAD and NADP), synthesis of important secondary metabolites, and the growth regulators cytokinins (Zrenner and Ashihara, 2011; Ashihara *et al.*, 2018; Witte and Herde, 2020). Therefore, purine nucleotides are key molecules for all living organism and its concentration is strictly controlled, through the regulation of its synthesis, salvage, and degradation pathway (Figure 3).

In mammals, the purine pool is mainly generated by salvage or recycling routes, while under abnormal physiological conditions that require higher demand for purines, such as in case of cancer cells, *de novo* synthesis pathway takes place (Pedley and Benkovic, 2017). On the contrary, in plants, purine nucleotides are mainly synthesized *de novo* and, particularly in ureidic legumes such as common bean, purine nucleotides are synthesized by the *de novo* pathway in the nodules, as precursors of the ureides (Schubert, 1986; Atkins *et al.*, 1997; Coleto *et al.*, 2016). There are 14 enzymatic reactions in *de novo* purine nucleotide synthesis pathway. The first reaction is performed by the phosphoribosyl pyrophosphate amidotransferase (PRAT), which catalyzes the formation of phosphoribosylamine (PRA) from glutamine and phosphoribosylpyrophosphate (PRPP) (Zrenner *et al.*, 2006; Coleto *et al.*, 2016; Voß *et al*., 2022). Subsequent reactions lead to the synthesis of the first purine nucleotide, inosine monophosphate (IMP). The last reactions consist of the transformation of IMP to guanosine monophosphate (GMP) through the action of IMP dehydrogenase and GMP synthetase, and to adenosine monophosphate (AMP) by the activity of SAMP synthetase and adenylosuccinase (Moffatt and Ashihara, 2002; Voß *et al.*, 2022.).

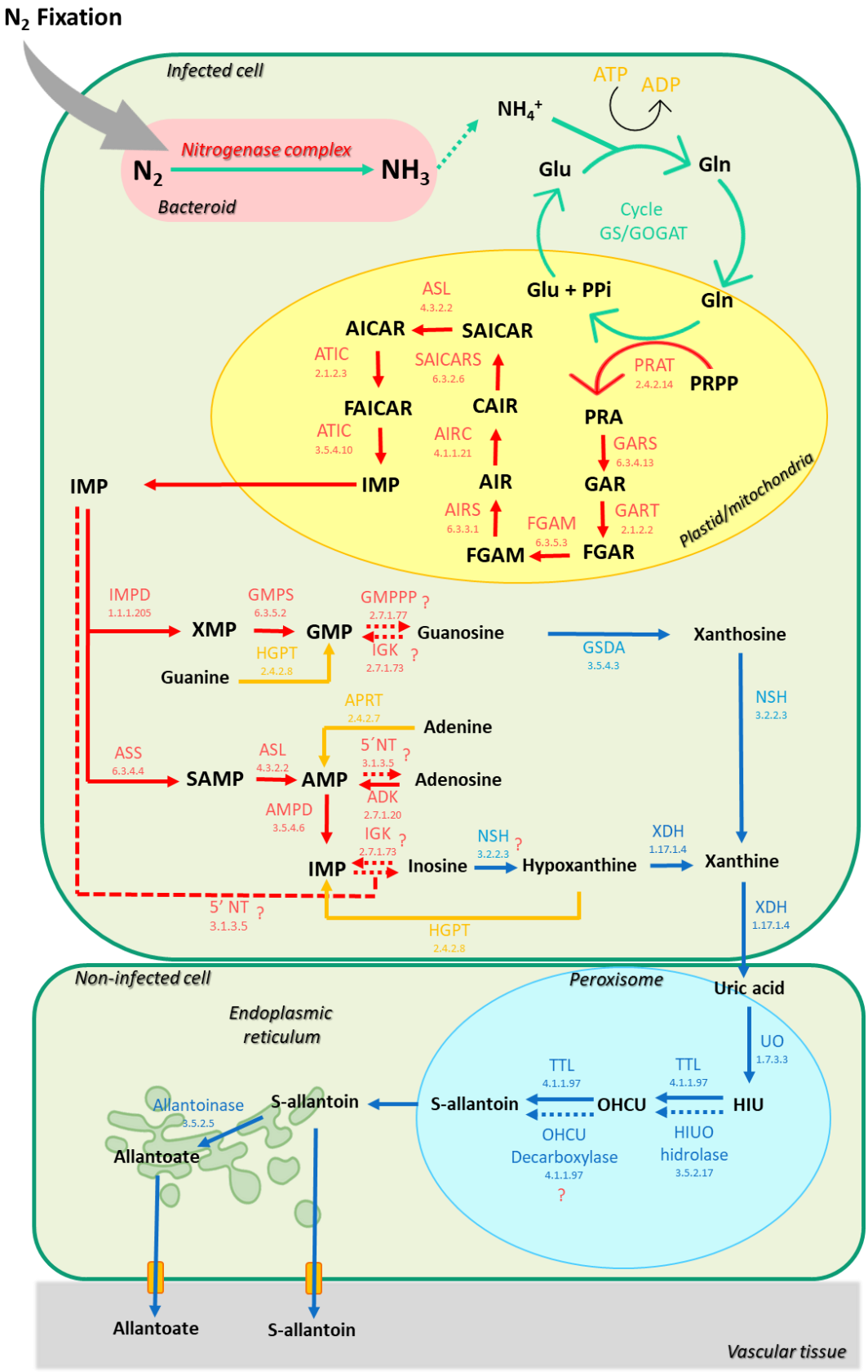

Figure 3. Diagram of the purine nucleotide regulation pathways in plant cells. *De novo* synthesis (red arrows), salvage (yellow arrows) and degradation (blue arrows) pathways of purine nucleotides and ureides formation in plant cells. Adapted from Moffatt and Ashihara, 2002; Voß *et al.*, 2022.

Afterwards, the degradation of purine nucleotides is carried out by their own dephosphorylation followed by the hydrolysis of nucleosides until they converge in the first common intermediate of the degradation pathway, xanthine (Zrenner *et al.*, 2006; Werner and Witte, 2011; Witte and Herde, 2020). Xanthine is converted into uric acid by the action of xanthine dehydrogenase (XDH). The uric acid is then oxidized to 5-hydroxyisourate (HIU) by the activity of the enzyme uricase. Later uricase product is converted to S-allantoin. The enzyme described as HIU hydrolase was initially found to hydrolyse 5-hydroxyisourate to 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) (Raychaudhuri and Tipton, 2002), and this product was later converted to S-allantoin by OHCU decarboxylase. However, it was later discovered that the transformation of 5-hydroxyisourate to S-allantoin was catalyzed by a single protein, named allantoin synthase, encoded by a transthyretin-like (TTL) gene in *Arabidopsis* (Figure 3) (Lamberto *et al.*, 2010; Pessoa *et al.*, 2010). S-allantoin is then transformed to allantoate by the enzyme allantoinase (allantoin amidohydrolase). Mainly allantoate, but also allantoin are used for long distance transport of N in ureidic legumes. Ureides can be metabolized to release CO_2 and NH_4^+ reassimilated to sustain plant growth (Werner and Witte, 2011; Díaz-Leal *et al*., 2012).

Although purine nucleotide can be synthesized *de novo* in most organisms (Marr and Müller, 1995), this pathway is energetically less favourable than the purine nucleotide recycling. Salvage pathway consists of the recycling of purine bases and nucleosides for purine nucleotide synthesis. This route is not only complementary to *de novo* synthesis of purine nucleotides, but it is essential in itself (Moffatt and Ashihara, 2002; Ashihara *et al.*, 2018). Even though the purine nucleotide recycling pathway is key to the correct development of animal and plant organisms, studies about this pathway in plants are very scarce.

Purine nucleotide salvage pathway

Salvage pathway acts in nucleotide formation using nucleobases and nucleosides from the environment or obtained from the metabolism, such as from the RNA turnover and depurination, and from reactions involving S-adenosylmethionine (SAM) among other (Girke *et al.*, 2014; Floyd *et al.*, 2015). There are four purine bases (adenine, guanine, hypoxanthine and xanthine) whose metabolic fate has been investigated in plant tissues. From them, the main precursor used in purine salvage is adenine followed by guanine.

Salvage of adenine nucleobase is carried out by the adenine phosphoribosyltransferase (APRT; EC 2.4.2.7) activity (Adams and Harkness, 1976; Deng and Ashihara, 2010*a*), whereas the salvage of guanine and hypoxanthine is done by the activity of hypoxanthine/guanine phosphoribosiltransferse enzyme (HGPRT; 2.4.2.8). This enzyme catalyzes the formation of GMP and IMP using PRPP and guanine or hypoxanthine, respectively, as substrates (Figure 4), while APRT enzyme catalyzes the formation of AMP using adenine and PRPP as substrates (Figure 4).

Salvage activity for hypoxanthine is lower in plants than that of adenine and guanine. (Katahira and Ashihara, 2006; Deng and Ashihara, 2010*b*; Yin *et al.*, 2014; Ashihara *et al.*, 2018). In mammalian tissues, the activity of the HGPRT enzyme is high and its deficiency causes the Lesch-Nyhan syndrome (Adams and Harkness, 1976; Nyhan, 1997). Although HGPRT activity was early found in extracts from higher plants (Le Floc'h and Lafleuriel, 1981), little research has been done on this enzyme in plants

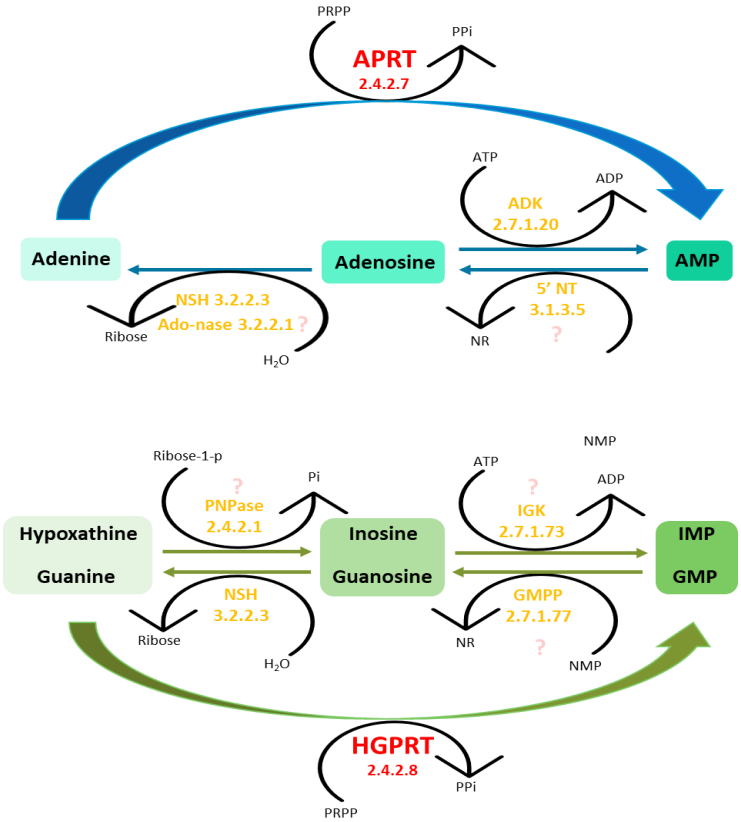

Figure 4. Scheme of the enzymes involved in the purine nucleotide salvage pathway. Enzymatic reaction catalyzed by Adenine phosphoribosyl transferase (APRT) and Hypoxanthine/guanine phosphoribosyl transferase (HGPRT), key enzymes in the purine nucleotide recycling pathway. Ado-nase (Adenosine nucleosidase); 5´NT (5´nucleotidase); PNPase (purine-nucleoside phosphorylase); ADK (adenosine kinase); NSH (Nucleoside hydrolase); GMPP (nucleotide phosphatase); IGK (inosine-guanosine kinase) Adapted from Moffat and Ashihara, 2002.

Characterization of *Arabidopsis* protein showed that HGPRT enzyme use guanine and hypoxanthine as substrates, but not xanthine (Katahira and Ashihara, 2006; Liu *et al.*, 2007; Deng and Ashihara, 2010*a*).

Despite the high relevance of HGRPT in mammalian tissues, in plants, HGPT activity levels are lower than those of adenine phosphoribosyltransferase (APRT; EC 2.4.2.7) activity (Adams and Harkness, 1976; Deng and Ashihara, 2010*a*). In humans, APRT deficiency leads to accumulation of 2,8-dihydroxyadenine (DHA), causing to the formation of crystalline nephropathy and kidney stones (Bollée *et al.*, 2012). In plants, APRT activity deficit causes *Arabidopsis* male sterility (Gaillard *et al.*, 1998). Furthermore, the functionality of APRT has not only been associated with the removal of adenine and adenosine, but also with the transformation of active cytokinin base to inactive cytokinin ribotides (Mok and Mok, 2001; Ashihara *et al.*, 2018), thus salvage of adenine not only restores adenine nucleotides, but also influence cytokinin homeostasis and plant development.

Justification and work hypothesis

Nitrogen and water are the two elements that most limit crop productivity globally. Most crops require the use of nitrogenous fertilizers, with the consequent economic and energy cost. Legumes represent a great advantage since they can establish symbiosis with soil bacteria, allowing legume crops to fix atmospheric nitrogen reducing fertilizers requirements. Thus, these crops would contribute to a more ecological and sustainable agriculture. However, although legumes can be cultivated under conditions of symbiotic nitrogen fixation, mainly because the symbiosis with rhizobia is particularly sensitive to adverse conditions, such as drought, or salinity (Araújo *et al.*, 2015), many farmers prefer to add fertilizers to ensure a good harvest. Therefore, studying the effects of drought on legume crops is particularly relevant in arid regions, where water availability is increasingly scarce.

Within the group of legumes, common bean belongs to the ureidic legumes group, which use the nitrogen fixed in the nodules for the synthesis of purine nucleotides. Then, purine nucleotide are oxidized to produce the ureides, allantoin and allantoate (Werner and Witte, 2011). Previous studies in our group revealed that common bean genotypes accumulate different concentrations of ureides depending on their degree of tolerance to water stress. Furthermore, it has been suggested that ureides could have a protective role

against drought and other types of stress, through regulation of ABA and possibly JA levels (Alamillo *et al.*, 2010; Coleto *et al.*, 2014).

The hypothesis of the current work is that plants cultivated under symbiosis should be more protected against water deficit than those fertilized with inorganic nitrogen, and that this protection is due in part to the ureides accumulated in them as molecules of storage and transport of nitrogen fixed in nodules. To demonstrate the proposed hypothesis, this thesis work addressed the three main objectives described below.

Objectives

The general objective of this work is to study the regulation at a molecular, biochemical, and physiological level of the response mechanisms of common bean plants against water stress, and specifically, the induction of routes affecting ureides production, as the induction of nucleotide degradation and nucleotide salvage pathways.

To address this general objective, two genotypes of common bean plants of contrasting drought resistance have been used (Figure 2): the PHA-0683 landrace (Monçao, Portugal), belonging to Cranberry commercial class of the New Granada race and Andean heritage, and the market class Great Northern "Mattherhorn" PMB-0220 breeding line, belonging to the Durango race, grown either under BNF conditions or fertilized with NO_3^-

To this end, the following specific objectives were set:

1. ***To compare the effects of water deficit and perform a large-scale analysis of the molecular changes that occur in common bean plants with high levels of ureides from symbiosis or in plants fertilized with nitrate and with low levels of ureides.***

This objective covers a wide and in-depth study that is presented in chapters 1, 2 and 3 of the current work. First, we show the results obtained by the transcriptomic analysis of common bean plants of the *P. vulgaris* PHA-0683 landrace subjected to 10 days of drought and cultivated under N_2 fixation. These results, addressed in Chapter 1, belongs to the first publication of this thesis work (López *et al.*, 2020*c*).

Then, the regulation of responses to water stress was compared between the drought-resistant genotype PHA-0683 and the commercial line Great Northern PMB-0220, with proven productivity and classified as drought intermediate tolerant. The results obtained in this part are reflected in Chapter 2 and in the second publication (López *et al.*, 2020*b*).

After comparing the response to water stress in two genotypes of common bean, this objective culminates in a more focused investigation on the commercial PMB-0220 line (Chapter 3). For this, a transcriptomic analysis was carried out comparing samples of *P. vulgaris* plants subjected to 10 days of drought but cultivated under N-fixation or inorganic N fertilization, and, therefore, that accumulated different concentrations of ureides. These results are showed in chapter 3 (López *et al.*, 2023).

2. ***Establish a cultural management protocol that improves the tolerance of bean plants to adverse conditions.***

To confirm whether the different sources of N, which in previous studies were shown to determine the concentration of ureides, could also influence drought tolerance and recovery in the market class common bean Great Northern PMB-0220. The work developed in this objective consisted of evaluating the recovery capacity of the common bean plant cultivated under N_2 fixation or fertilized with NO_3^-, after 10 days of drought. To do this, after the water stress treatment, the plants were watered again until harvest. This objective complements the analysis developed in the last part of objective 1, raising a more agronomic perspective. The results have been included in the publication of Chapter 3 (López *et al.*, 2023).

3. ***Molecular and biochemical characterization of the role of nucleotide degradation and ureide synthesis in mobilization and recycling processes in stress situations.***

The aim of objective 3 is to characterize the adenine phosphoribosyltransferase (APRT) enzyme, which participate in the purine nucleotide salvage pathway and could be relevant in the ureide synthesis and in plant development. The results obtained from this objective are presented in Chapter 4 (pending publication) of this work. However, part of the methods used in Chapter 4 and the current objective 3 has been published in a review (Alamillo *et al.*, 2023)

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CHAPTER 1

TRANSCRIPTOMIC RESPONSE TO WATER DEFICIT REVEALS A CRUCIAL ROLE OF PHOSPHATE ACQUISITION IN A DROUGHT-TOLERANT COMMON BEAN LANDRACE

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Chapter 1. Transcriptomic response to water deficit reveals a crucial role of phosphate acquisition in a drought-tolerant common bean landrace.

Abstract

Drought is one of the most critical factors limiting legume crop productivity. Understanding the molecular mechanisms of drought tolerance in the common bean is required to improve the yields of this important crop under adverse conditions. In this work, RNA-seq analysis was performed to compare the transcriptome profiles of drought-stressed and well-irrigated plants of a previously characterized drought-tolerant common bean landrace. The analysis revealed responses related with the abscisic acid signaling, including downregulation of a phosphatase 2C (PP2C) and an abscisic acid-8' hydroxylase, and upregulation of several key transcription factors and genes involved in cell wall remodeling, synthesis of osmoprotectants, protection of photosynthetic apparatus, and downregulation of genes involved in cell expansion. The results also highlighted a significant proportion of differentially expressed genes related to phosphate starvation response. In addition, the moderate detrimental effects of drought in the biomass of these tolerant plants were abolished by the addition of phosphate, thus indicating that, besides the ABA-mediated response, acquisition of phosphate could be crucial for the drought tolerance of this common bean genotype. These results provided information about the mechanisms involved in drought response of common bean that could be useful for enhancing the drought tolerance of this important crop legume.

Introduction

Common bean (*Phaseolus vulgaris*) is the most important legume for human consumption worldwide. It is grown throughout the world, especially in developing countries, with a large economic and social impact (Broughton *et al.*, 2003; Beebe *et al.*, 2013; De Ron *et al.*, 2015). Bean cultivation can be done in the absence of nitrogen fertilizers under conditions of symbiotic nitrogen fixation, thus reducing the economic and environmental impact of fertilization. However, nitrogen fixation in common beans is not usually very efficient (Graham and Vance, 2003), mainly because symbiosis with N_2-fixing rhizobia is particularly sensitive to adverse conditions, especially to water scarcity (Sinclair and Serraj, 1995*a*; Serraj *et al.*, 1999*c*; Araújo *et al.*, 2015). It is estimated that between 60 and 73% of this crop is grown in areas that suffer from drought

conditions (Beebe *et al.*, 2008), and this problem is expected to worsen due to climate change (McClean *et al.*, 2011). In common beans, drought inhibits nitrogen fixation even earlier than photosynthesis. Besides limiting fixed nitrogen supply, drought also affects the absorption of mineral nutrients and translocation of assimilates, resulting in a drastic reduction in yield (Rosales-Serna *et al.*, 2004; Beebe *et al.*, 2013; Etienne *et al.*, 2018). Nevertheless, due to their high dissemination and diversity, common beans exhibit enormous genotypic variability in their drought tolerance (Frahm *et al.*, 2004; Beebe *et al.*, 2008, 2013). Drought tolerance has been evaluated in many bean germplasm collections, revealing the complexity of the trait, which has additive and quantitative effects, and very significant interaction with the environment (Terán and Singh, 2002; Muñoz-Perea *et al.*, 2006), thus limiting the obtention of highly drought-tolerant cultivars.

Common bean belongs to the so-called ureidic legumes, which incorporate the nitrogen fixed in the nodules for the *de novo* synthesis of purine nucleotides (Coleto *et al.*, 2016), which are oxidized to produce the ureides, allantoin, and allantoate (Zrenner *et al.*, 2006). In these legumes, ureides are the main nitrogen transport and storage molecules (Atkins *et al.*, 1982; Díaz-Leal *et al.*, 2012). Ureide production also increases under stress conditions as a result of the degradation of nucleotides. Recently, there have been several studies showing that ureide production could be beneficial for plants subjected to adverse conditions (Brychkova *et al.*, 2008; Alamillo *et al.*, 2010; Irani and Todd, 2016; Nourimand and Todd, 2017). However, the accumulation of ureides has been considered for a long time as a symptom of the drought sensitivity of ureidic legumes since these compounds increase particularly in the most sensitive varieties of soybean and common bean (Serraj *et al.*, 1999*b*; King and Purcell, 2005; Alamillo *et al.*, 2010). In our group, the capacity to tolerate drought stress of four common bean genotypes was compared at the physiological and biochemical levels. We found that drought stress caused an increase in ureides in the sensitive common bean varieties but not in the tolerant ones (Coleto *et al.*, 2014). In addition, among the compared genotypes, landrace PHA-683 behaved like a very tolerant one, with only mild symptoms of water deficit appearing after two weeks of water withholding. This genotype was able to maintain unaltered N_2 fixation after 7 days without irrigation, and the nodule activity was only partially inhibited after two weeks of stress. Moreover, these plants did not accumulate ureides, even after two weeks of stress (Coleto *et al.*, 2014).

Most efforts to obtain drought-tolerant legumes have been made using a phenotypic or genotypic characterization, based on the analysis of a discrete number of parameters. However, molecular analyses are required to understand how drought tolerance is achieved in ureidic legumes. In recent years, transcriptome sequencing has emerged as a powerful tool for providing high-resolution data and transcription networks widely applied in the analysis to developmental or environmental responses in many crops, including some legumes (Chen *et al.*, 2013; Cabeza *et al.*, 2014; Jha *et al.*, 2020), but only a few transcriptome analyses have considered the tolerance to drought in common bean (Wu *et al.*, 2014, 2016, 2017).

The hypothesis of this work was that investigating the molecular mechanisms of drought tolerance in common bean genotypes known to exert high tolerance levels would help to discover key factors that could be used in the amelioration of abiotic stress effects in this important crop.

We used the common bean PHA-0683 landrace, recently shown to maintain active nitrogen fixation and to retain high relative water content in their tissues, until severe water stress was imposed (Coleto *et al.*, 2014). To decipher the molecular changes associated with drought tolerance, the genome-wide expression analysis using RNA-seq in response to drought in PHA-0683 plants was done. The analysis of the differentially expressed genes (DEGs) and the functional gene ontology (GO) enrichment between control and drought-stressed plants revealed a prevalent relevance of genes related to phosphate nutrition stress in response to water deficit of this tolerant genotype.

Material and methods

Plant Material and Growth Conditions

In this study, a previously characterized drought-tolerant landrace PHB-0683 common bean (*Phaseolus vulgaris L.*), originated in Moncao (Portugal), was used (Coleto *et al.*, 2014). Seeds were kindly provided by Prof. A. de Ron, from the Misión Biológica de Galicia's seed collection (Pontevedra, Spain). Seeds were soaked in 96% ethanol for 30 s. Ethanol was discarded, and seeds were immersed in 5% sodium hypochlorite for 5 min. Then, seeds were repeatedly washed 6 times with sterile water and placed on moist paper on 120 mm Petri dishes for their imbibition at 26°C and dim illumination during 72 h. After germination, three seedlings were sown on each pot (16 cm diameter, 18 cm height) filled with a mixture of vermiculite/perlite mixture (2/1 w/w) and inoculated with a fresh

suspension of *Rhizobium leguminosarum* ISP 14, which had been cultured at 28°C for less than 30 h. Inoculated plants were watered three times a week with nitrogen-free nutrient solution (Rigaud and Puppo,1975). Plants were grown in a culture chamber with 300 $\mu E.m^{-2}. s^{-1}$ lighting for 16 h at 26°C and 8 h of darkness at 20°C and relative humidity of 70%, under well-irrigated conditions for four weeks, as previously described (Coleto *et al.*, 2014). Four weeks after sowing, the plants were randomly separated into two sets, and the irrigation was withheld from one-half of the pots, and the second half was regularly watered with the standard nutrient solution to serve as controls.

Soil water capacity (SWC) was determined gravimetrically. Basically, pots filled with substrate were watered to excess, left to drain, and weighed to estimate the 100% SWC at sowing. The weighing was repeated during the drought treatments for both control and drought-stressed plants. SWC was maintained at 80%–90% for control plants during the whole experiment. Leaf relative water content (RWC) was estimated as RWC (%) ((Fw − Dw)/(Sw − Dw)) × 100. The water-saturated weight (Sw) of leaf samples was obtained by keeping leaf disks in distilled water at 4°C for 12 h. Then, the samples were oven-dried at 70°C to get a constant dry weight (Dw) (Coleto *et al.*, 2014).

Effect of Phosphate Addition on the Responses to Drought Stress

Plants under the condition of atmospheric nitrogen fixation were cultured and irrigated with the standard nitrogen-free nutrient solution containing 80 µM phosphate (normal P) until plants were 21 days old. Then, half of the plants were watered three times (in alternate days) with nutrient solution containing 200 µM phosphate (high P) for a week, whereas the second group was maintained under irrigation with the normal P solution. P-supplemented and control plants at 28 days old were randomly separated into two groups, one that received no further watering for 10 days (drought treatment), and the other that was irrigated with the regular nutrient solution (control).

RNA-Seq Analysis

Plants cultured under standard nitrogen-fixing conditions for four weeks were randomly distributed into two groups, one of which was submitted to 10 days of water deprivation, whereas the group used as a control was regularly irrigated with the standard nitrogen-free nutrient solution. Three biological replicates, each consisting of the pooled 4th trifoliate leaves from 3–4 plants, from at least 3 independent control pots and three drought-treated pots, were used for RNA-seq analysis. Total RNA was isolated from the

6 samples by using Pure-link RNA isolation Kits (Thermo Fisher; Spain), according to the manufacturer's instructions. RNA was quantified in a Nanodrop, and its quality was assessed in a 2100 bioanalyzer (Agilent). Poly A mRNA was isolated from 5 µg total RNA from each sample using Ambion Dynabeads™ mRNA Purification Kit (Thermo-Fisher) and used for cDNA libraries preparation using the Ion Total RNA-Seq Kit v2 for whole transcriptome libraries (Life Technologies Corporation, California, USA). cDNA libraries were loaded by an Ion Chef System, in three Ion 540 sequencing chips (each containing cDNA libraries from one control and one treated sample), and then further sequenced using an Ion S5 System (Thermo-Fisher Scientific). RNA-seq yielded approximately 33–37 million reads per library. The raw reads were analyzed for quality by FastQC (Andrews and Babraham, 2010) and processed to filter out poor quality sequences (Cutadapt version 1.9 (-m 100) and BBDuk version 35.43 (qtrim = rt trimq = 20)). The generated clean data were aligned to the reference genome for *P. vulgaris L.* v2.1 obtained from the Phytozome website (http://www.phytozome.net/) (Schmutz *et al.*, 2014; Ghosh and Chan, 2016). Genes were ranked according to normalized fragments per kilobase per million mapped reads (FPKM) to identify differentially expressed genes (DEGs). FPKM values were assigned to each gene by comparing the FPKM value under the drought treatment to that in the control condition. DegSeq2 R package was used to identify differentially expressed genes. Genes that were up- or downregulated at least 2-fold change (log2) with false discovery rate (FDR) adjusted p-value ≤ 0.05 were considered as DEGs (Love *et al.*, 2014; Lowe *et al.*, 2017).

GO Enrichment Analysis of DEGs

The bioinformatics analysis of DEGs was performed using Blast, Uniprot, and AgriGo v2.0 software (http://systemsbiology.cau.edu.cn/agriGOv2/) (Tian *et al.*, 2017) to determine the biological process, molecular functions, and cellular components enriched in the drought-treated samples.

Validation of DEGs by qRT-PCR Analysis

To validate RNA-Seq results, sixteen genes were selected from the list of DEGs and subjected to quantitative RT-PCR analysis. Gene-specific primer pairs (Supplementary Table S2) were designed by using Primer 3 + software and the qPCR default setting (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The total RNA was isolated using RNA-zol, according to the manufacturer's instructions, and treated with

RNase-free DNase I (New England Biolabs) at 3°C for 10 min to eliminate polluting genomic DNA from samples. Next, first-strand cDNA synthesis was done from 2.5 µg of DNase-treated RNA using PrimeScript™ reverse transcriptase (TaKaRa) following the manufacturer's instructions.

The expression analysis was carried out by qRT-PCR in an iCycler iQ System (Bio-Rad) using iQ SYBR-Green Supermix (Bio-Rad) and the specific primers for each gene (Supplementary Table S2). The program used consisted of an initial denaturation, together with a Taq polymerase activation, at 95°C for 5 min followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and, finally, 80 cycles of 30 s at 6°C. The relative expression of each gene in control and drought-stressed samples was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen; 2001), normalized to that of *Actin-2*. The quantification was performed using three independent biological replicates.

Promoter Analysis of the Phosphate-Related DEGs

The 5′ upstream regions (1.5 kb DNA sequence of each gene; Supplementary Table S3) were obtained from Phytozome database v12, and cis-elements scan was done using plant CARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot *et al.*, 2002).

Determination of Pi Concentration

Pi concentration was determined in leaf samples from control and drought-stressed plants cultured under standard P nutrition or which received a higher P concentration. The extraction protocol from leaf tissues was used as described in (Chiou *et al.*, 2006). The Pi content was determined according to (Ames, 1966). In brief, 50 mg leaf tissue was homogenized in 10 µl.mg^{-1} of extraction buffer pH 8 (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 1 mM β-mercaptoethanol, 1 mM PMSF). Then, samples were centrifuged at 11,000 *g* for 10 min, and 100 µl of the supernatants were mixed with 900 µl of 1% glacial acetic acid and incubated for 30 min at 42°C. For Pi measurement, 300 µl of the extract was collected in a new tube to which 600 µl of molybdate solution (1 N H_2SO_4 and 0.42% NH_4MoO_4) and 100 µl of reducing solution (10% ascorbic acid) were added. Finally, the mixture was incubated at 45°C for 20 min, and the absorbance at 820 nm was determined. The Pi concentration was obtained according to the calibration curve using known Pi concentrations.

Experimental Design and Statistical Analysis of The Data

A total of 18 plants were randomly divided between control plants and plants subjected to 10 days of drought for the RNA-seq drought experiment. Three independent biological replicates per condition, each from three independent plants, were used for the RNA-seq analysis. The whole experimental design from the other 18 plants was repeated to obtain the three biological replicates used in the qRT-PCR validation of RNA-seq DEGs.

In addition, another independent experiment was done in which 21 days old plants were separated into two groups, one of which was irrigated with nutrient solution enriched in P for one week. Then, the irrigation was withheld for 10 days for half of the 28 days old plants from the low and high P groups. Three replicas of each condition were used. Each replica consisted of a total of three plants per pot for each condition. Statistical analysis was done by Student's *t-test* and ANOVA using GraphPad Prism 6 software package.

Results

To elucidate the molecular strategies displayed by common bean landrace PHA-683 to tolerate drought conditions, the RNA-seq approach was chosen to compare the genome-wide changes in transcript levels in response to water deficit. Since, in our previous work, these plants did not show any apparent drought symptoms after 7 days of water stress (Coleto *et al.*, 2014), 10 days of water deprivation was chosen as the optimal stress length to investigate the changes in gene expression associated with early events of water deficit in these plants. Plants cultured under N_2 fixation conditions were regularly irrigated until they were 28 days old, and then they were randomly distributed into two groups, one kept under regular watering, whereas the other one received no further irrigation for 10 days. Total RNA from three independent biological replicates from each treatment was used to obtain the mRNA fraction from control and 10-days-drought-stressed trifoliate leaves. The mRNA was then copied and amplified into six independent cDNA libraries. The transcriptome changes of control and drought-stressed leaves were examined using the Ion-Torrent RNA-Seq technology. The total number of raw, pre-processed reads ranged from 32 to 37 million reads per library, with an average length of 135 bp. After the removal of low-quality reads and adapter sequences, clean reads were aligned with the *P. vulgaris L.* v2.1 reference genome obtained from the Phytozome website (http://www.phytozome.net/) (Schmutz *et al.*, 2014). The expression levels of the genes in leaf samples from 10 days-drought-stressed were compared to control well-irrigated

plants, and a total of 211 differentially expressed genes (DEGs) were found, with a Log2FolChange > 1, and a p-value adjusted to multiple testing < 0.01 (Supplementary Table S1).

Functional Annotation and Gene Ontology Enrichment of DEGs upon Drought Stress

Among the annotated genes, showing significant differential expression in drought and control samples, 47% were found upregulated, and 53% of DEGs appeared downregulated (Figure 1A). To find out a biological significance of DEGs during drought, we made a gene ontology (GO) enrichment analysis of up- and downregulated genes in relation to molecular function, biological process, or cellular component using AgriGO v2.0 (Tian *et al.*, 2017) (Figure 1a–d). Among the molecular function enriched terms, the most prevailing ones were those related to hydrolase activity, with phosphatase, followed by glycosyltransferase and endopeptidase activities (Figure 1b). According to the biological process, the most enriched one concerned carbohydrate or polysaccharide metabolic processes (Figure 1c). The most enriched cellular component was the extracellular region, including apoplast and cell wall components, followed by thylakoid membranes (Figure 1d).

To further dissect the overrepresented molecular functions that change among drought and control samples, the number of induced and repressed genes were depicted (Figure 2). Interestingly, the highest proportion of induced genes corresponded to phosphatase activity categorization, whereas repressed genes were mostly those of transferase and glycosidase activities. The closest view of the enriched molecular functions revealed that, besides phosphatases, there were also a significant number of phosphate homeostasis-related genes, including proteins involved in phosphorous nutrition, which were either induced or repressed in response to the drought treatment according to the RNA-seq results (Supplementary Table S1), revealing that the regulation of phosphate homeostasis was a crucial event in response to drought stress in this common bean landrace.

Interestingly, besides the phosphate-related genes, 6% of the DEGs were involved in cell wall modification, including two coding for a cellulose synthase H1—*Phvul.005G117833* and *Phvul.005G116501*—that were induced 9.8 and 3.2 fold, respectively, in the drought-treated samples and several downregulated genes coding for extensins (*Phvul.004G161500*) and for xyloglucan endotransglucosylase hydrolases belonging to

expansins family (eight genes), thus indicating that drought caused important changes in the cell wall structure.

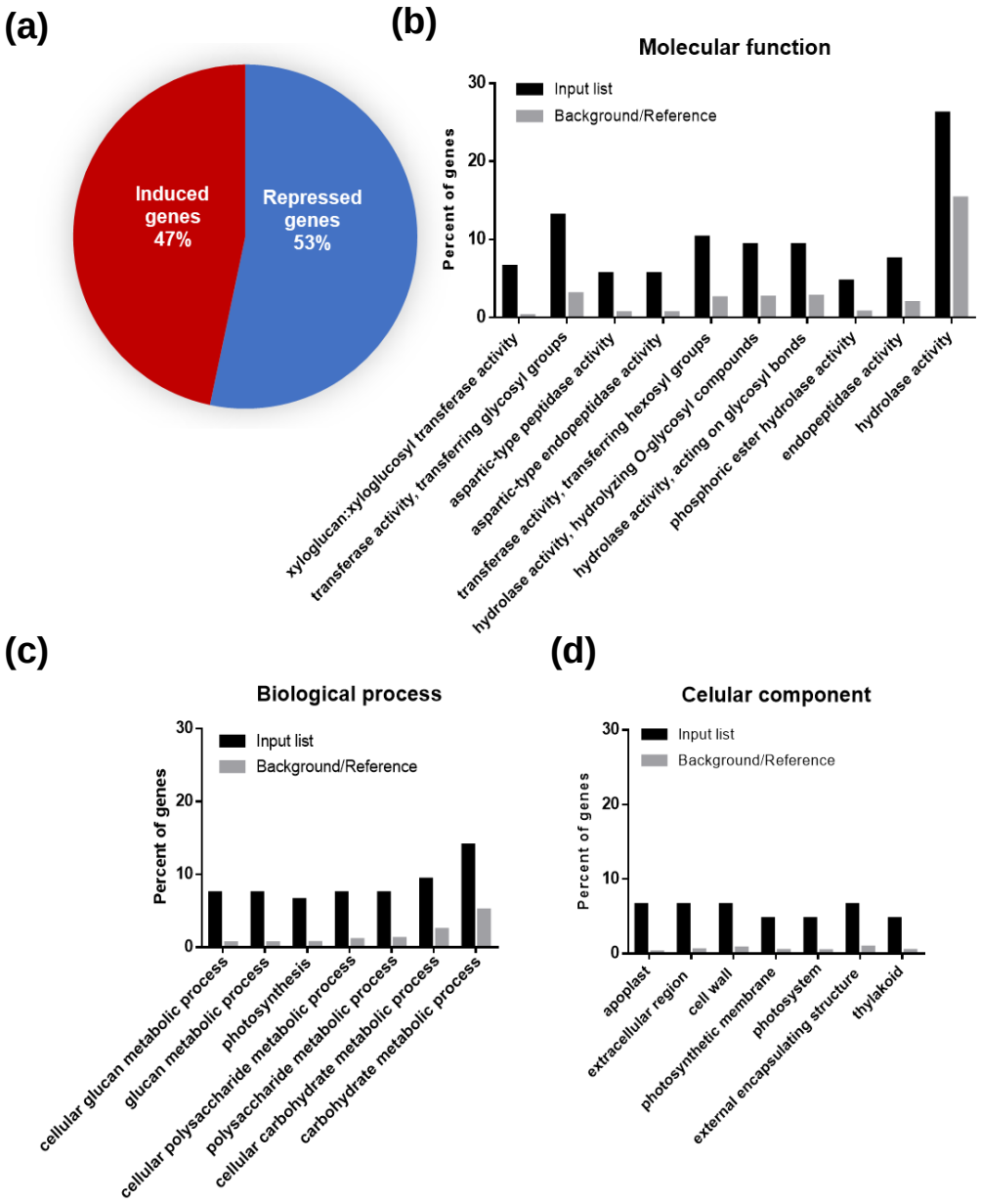

Figure 1. Analysis GO enrichment of the DEGs. (a) Diagram showing percentage (%) of induced (red) and repressed genes (blue) in *P. vulgaris* leaves in response to drought. (b) Molecular functions, (c) biological process, and (d) cellular component gene ontology (GO) enrichment categorization of the differentially expressed genes in RNA-seq analysis using the AgriGOv2.0 software (http://bioinfo.cau.edu.cn/agriGO/)

Among the induced glycosyltransferases, there was also one galactinol synthase (*Phvul.007G203400*) involved in the biosynthesis of the raffinose family oligosaccharides (RFOs) that function as osmoprotectants (Taji *et al.*, 2002).

On the other hand, 4% of the DEGs was found to belong to transcription factors (TFs), including the induction of a WRKY (*Phvul.007G046800*) and a MYB (*Phvul.003G028000*) and the downregulation of six putative TFs belonging to the MYB, NAC, and LHDH families. Moreover, 6% of DEGs were found to correspond to kinases, phosphatases, calcium-binding, and protein receptors that could be involved in the early signaling of the stress responses. Among these, there was a downregulation of a putative phosphatase 2C (*Phvul.001G021200*). Downregulation was also found for a putative abscisic acid 8′-hydroxylase (*Phvul.002G122200*), involved in the degradation of abscisic acid (ABA), thus suggesting the upregulation of ABA-mediated responses.

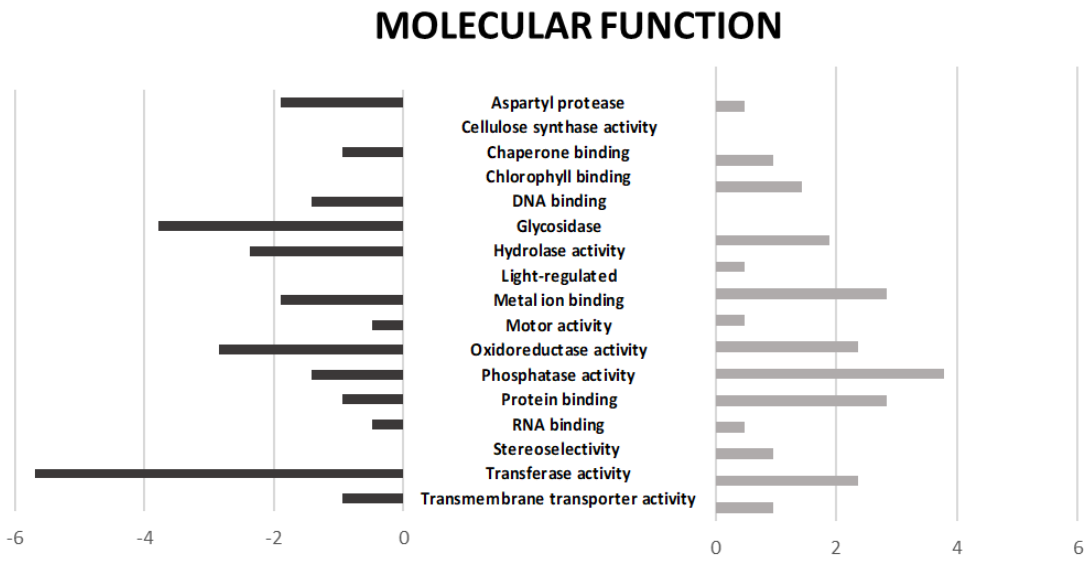

Figure 2. Molecular function of the DEGs. Molecular function depicted as a percentage of induced (grey bars) and repressed (black) differentially expressed genes.

There was also a significant proportion of genes related to photosynthesis, including several light-harvesting, chlorophyll-binding proteins, which could be related to the protection of photosynthetic complexes, as well as several stress-related genes, such as glutathione S-transferase, small heat shock proteins, chaperones, and others. Finally, 12% of the DEGs encoded putative proteins of unknown functions.

Validation of Changes in the Expression Levels by qRT-PCR

Real-time quantitative PCR was conducted using gene-specific primers (Supplementary Table S2) to validate the expression patterns revealed by the RNA-seq analysis. Sixteen genes were selected from the list of DEGs, and the relative expression of target genes was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) as the mean ± sd from the results of three independent biological replicates. As shown in Figure 3a, results found in

the RNA-seq analysis fully correlated with those found in the qPCR (R square of 0.92) for all the selected genes. Among the DEGs whose pattern of expression was validated by qRT-PCR, were the genes related to ABA responses, as the PP2C (*Phvul.001G021200*) and the putative ABA 8′-hydroxylase gene (*Phvul.002G122200*) appeared as repressed in the drought condition. The expression of the WRKY 70 (*Phvul.008G185700*) and the MYB (*Phvul.003G028000*) transcription factors upregulated upon the drought stress in the RNA-seq and was also induced in the qRT-PCR.

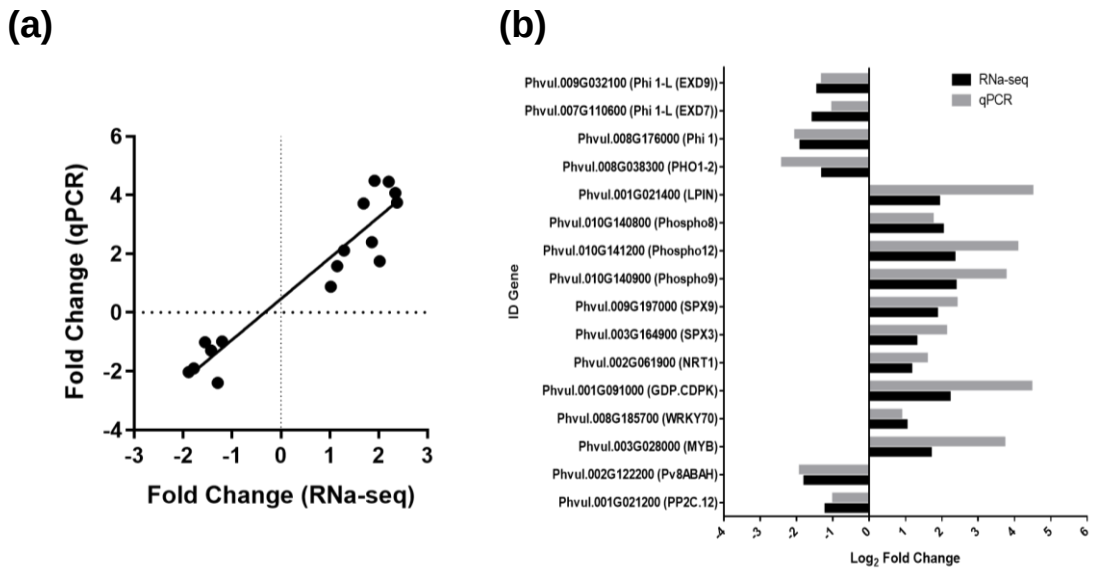

Figure 3. Validation by qRT-PCR of the changes in expression in response to drought of several genes identified in the RNA-seq. (a) Pearson correlation between RNA-seq and qRT-PCR gene expression values of selected differentially expressed genes (DEGs). (b) Graphical representation of overexpressed (positive fold change) and the repressed (negative fold change) genes: *Phvul.001G021200 (PvPP2C.12); Phvul.002G122200 (Pv80ABAH); Phvul.003G028000 (PvMYB); Phvul.008G185700 (PvWRKY70); Phvul.001G091000 (PvGDP.CDPK); Phvul.002G061900 (PvNRT1); Phvul.003G164900 (PvSPX3); Phvul.009G197000 (PvSPX9); Phvul.010G140900 (PvPhospho9); Phvul.010G141200 (PvPhospho12); Phvul.010G140800 (PvPhospho8); Phvul.001G021400 (PvLPIN); Phvul.008G038300 (PvPHO1-2); Phvul.008G176000 (PvPhi 1); Phvul.011G004400 (PvPhi 1-L (EXD7)); Phvul.009G032100 (PvPhi 1-L (EXD9)).*

A relevant amount of RNA-seq DEGs appeared as related to phosphate (P) nutrition; therefore, the expression levels of several of the phosphate-related genes were included in the list of genes whose changes in expression levels were validated by qRT-PCR. Among them, there were three genes coding for pyridoxal phosphate or PDX-related protein phosphatases, belonging to the Phospho1 or PS2 inorganic pyrophosphatase 2-like gene family (Denslow *et al.*, 2007); we named the genes as Phospho8

(*Phvul.010G140800*), Phospho9 (*Phvul.010G140900*), and Phospho12 (*Phvul.010G141200*), which were induced in the RNA-seq and also appeared as highly induced in the qRT-PCR analysis (Figure 3b). There were also two genes related to the phosphorous starvation sensing or SPX domain proteins (Wild *et al.*, 2016), which we named SPX9 (*Phvul.009G197000*) and SPX3 (*Phvul.003G164900*). According to the qRT-PCR results, the expression level of these genes was also heavily induced in the drought-stressed samples (Figure 3b).

The induction was also found for PvLPIN (*Phvul.001G021400*), a putative phosphatidate phosphatase that has been related to critical responses to phosphate starvation (Nakamura *et al.*, 2009), and for PvGDP-CDPK (*Phvul.001G091000*), a glycerophosphodiester phosphodiesterase related to a multifunctional cyclin-dependent kinase, putatively involved in maintaining cellular phosphate homeostasis under phosphate starvation (Cheng *et al.*, 2011) (Figure 3b). In contrast, downregulation was found for the phosphate transporter-related gene, PvPHO1-2 (*Phvul.008G038300*) (Figure 3b), and several genes coding phosphate-induced genes as PvPHI and PvPHI-like belonging to the EXORDIUM like protein family (Schröder *et al.*, 2009, 2011), from which two of them are shown in Figure 3b (*Phvul.007G110600; Phvul.009G032100*). Moreover, there was upregulation of the dual nitrate transporter NRT1 (*PvNRT1, Phvul.002G061900*) (Figure 3b), which has recently been described as an integrator of nitrate and phosphate signaling networks (Wang *et al.*, 2020).

Expression changes of Phosphorous homeostasis related genes in Drought-Stressed Plants upon Phosphorous Supplementation

To investigate if phosphate starvation was the key factor for the induction of phosphate-related genes in the drought-stressed plants, an experiment was performed in which a group of plants was supplemented with a higher concentration of P in the irrigation solution for one week just before the water deficit treatment, and the effect of phosphate addition was analyzed.

Control and water-stressed plants with and without the addition of a higher concentration of P were collected 10 days after the drought treatment, and the expression of a group of the phosphate-related genes was determined. As shown in Figure 4a–c, the expression of the three inorganic pyrophosphates (Phospho 8, 9, and 12) of the Phospho1 family was induced to similar levels in the drought-stressed samples from plants irrigated with the

lower (standard P levels) and with the higher P concentration, thus suggesting that drought was more determinant than P availability for the induction of these genes.

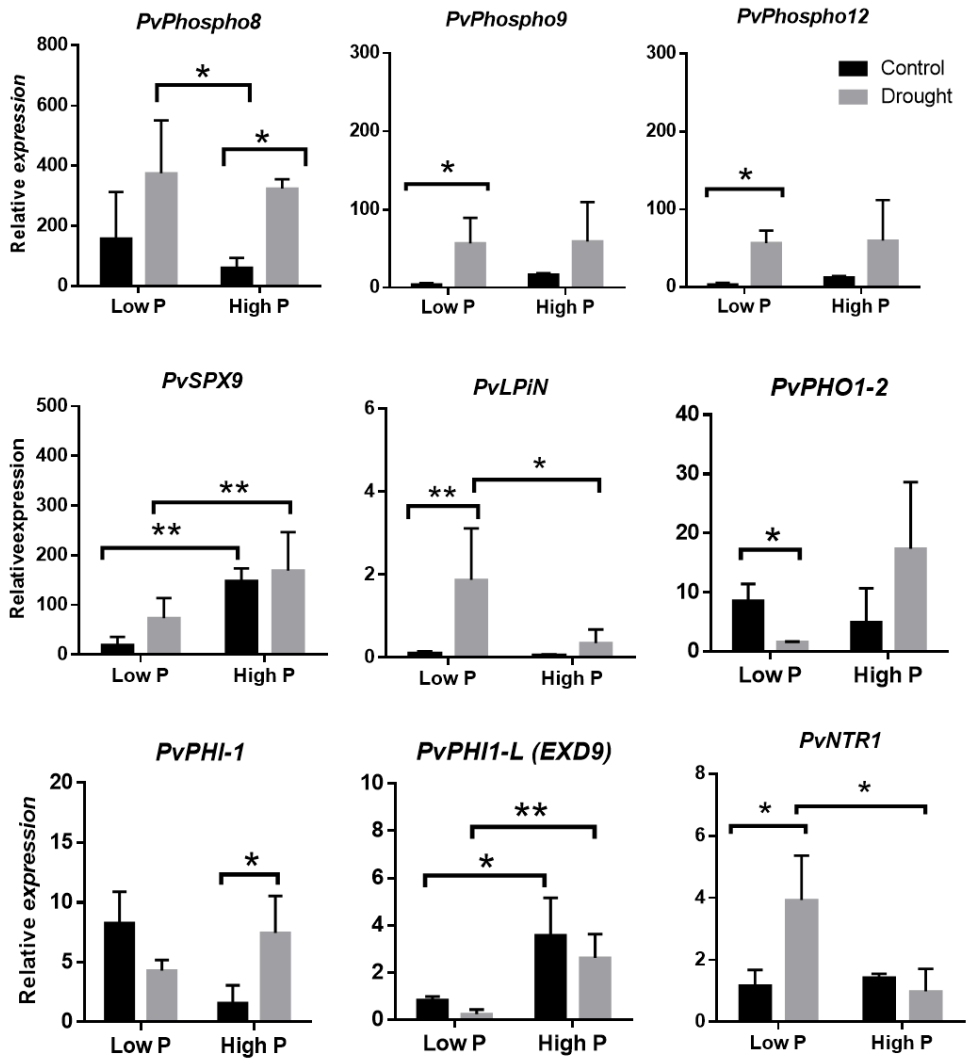

Figure 4. Relative expression by qRT-PCR. The relative expression of phosphate starvation-related stress genes in leaves from control and 10 days-drought-stressed plants that were grown under symbiotic nitrogen fixation conditions with 82 µM of KH_2PO_4 (Normal P) or received 200 µM of KH_2PO_4 (High P) for one week, just before the drought treatment. (a–c*)* Relative expression of *PvPhospho8, PvPhospho9, PvPhospho12;* (d) *PvSPX9;* (e) *PvLPIN*; (f) *PvPHO1-2;* (g*) PvPHI-1;* (h) *PvPHI1-L or EXORDEUM 9;* (i) *PvNTR1* expression in response to P supplementation and drought. Data are means of three independent experiments. Asterisks indicate statistically differences *(* $p < 0.05$*) and (** $p < 0.005$*).*

In contrast, the supplement of phosphate prior to the drought treatment significantly attenuated the induction of the phosphatidate phosphatase (*PvLPIN*) gene (Figure 4e), suggesting that P limitation was the main condition for the upregulation of this gene. In addition, the *SPX 9*, and the phosphate-induced genes *PHO1-2*, *PHI1*, and *PHI-L* (EXD 9) (Figure 4f–h) did also show significant differences in response to drought among the normal and the high P conditions. The addition of phosphate was able to mitigate the

downregulation caused by the water stress of several of the phosphate responsible (*PHI and Phi-like*) genes, suggesting that phosphate cellular level was the key regulatory factor for these genes. Interestingly, the expression of the dual nitrate transporter *NTR1.1* was not induced in response to drought in the high phosphate samples, indicating that phosphate level also controlled the expression of *PvNTR1.1* (Figure 4i).

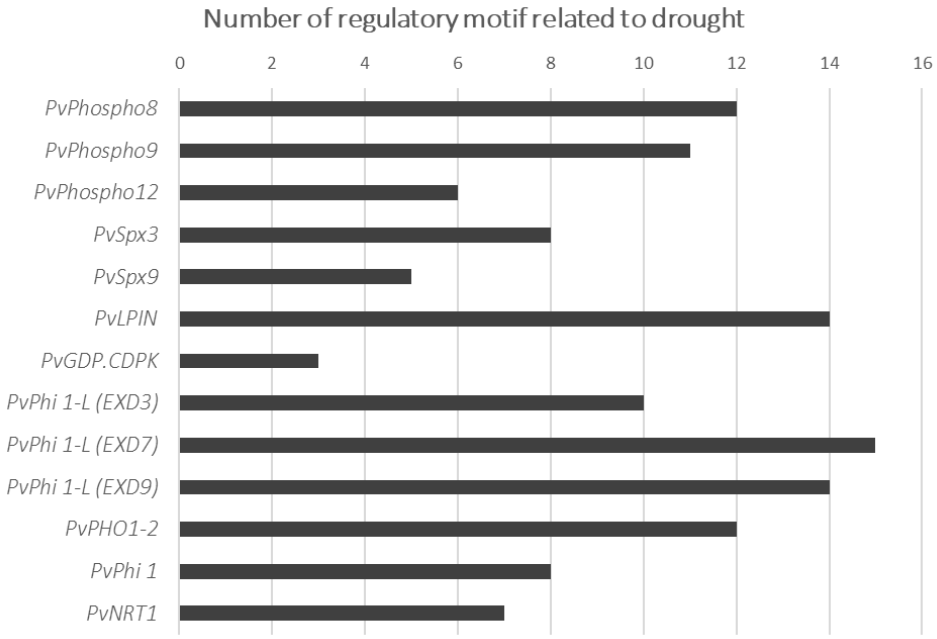

Figure 5. Representation of the number of regulatory motifs related to drought in the promoter sequences of the phosphate starvation related DEGs. Promoter sequences of the genes were retrieved from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#), and 1500 bp upstream the ATG of each gene was analyzed using the PlantCARE bioinformatics platform of plant regulatory motives search (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)

In order to further investigate why the expression of several phosphate-related genes was mainly regulated by water deficit, a search for water-stress cis-regulatory motives on the upstream genomic sequences of these genes was done using Plant Care software (Lescot *et al.*, 2002). As shown in Figure 5, the search for regulatory motives present in the -1500 bp upstream the ATG of the promoter regions of the investigated genes revealed a significant number of drought, ABA, or osmotic stress-related motives (Supplementary Table S3), ranging from 3 motives in the promoter of the glycerophosphodiester phosphodiesterase (PvGDP-CDPK) up to 15 motives in the upstream sequence of LIPIN coding gene (Figure 5 and Supplementary Table S3).

Physiological Effects of Phosphorous Supplementation on Drought-Stressed Plants

In addition to the molecular response to drought, we also determined how much of the phosphate supplementation reached the shoots of the treated plants. The content of Pi was about twice higher in phosphate-supplemented well-irrigated plants. As expected, drought drastically reduced the amount of Pi in the leaves of the treated plants, although it was significantly higher in the drought-stressed plants that received the Pi supplement compared to the samples grown under standard P (Figure 6a). Drought reduced the Pi content to about 50% in both the low and the high P samples, although in the latter, the available Pi remained higher than the one in control irrigated samples of the low P (Figure 6a).

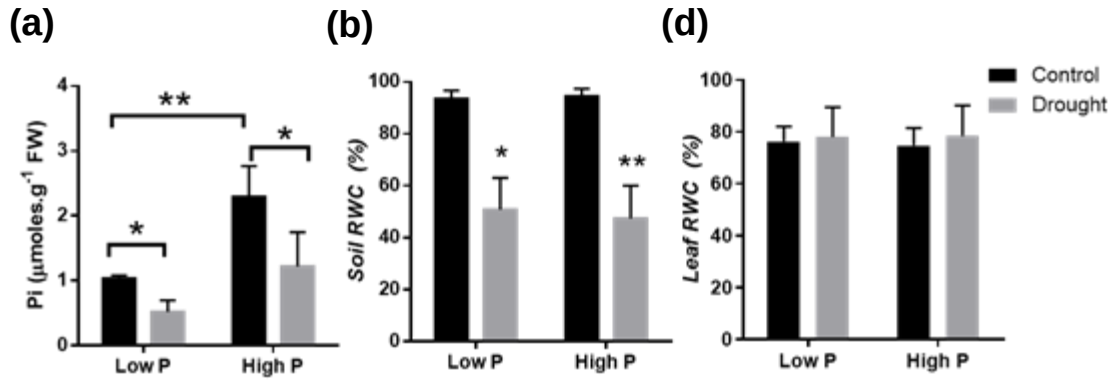

Figure 6. Determination of the concentration of P and RWC in drought experiments. (a) Determination of inorganic phosphorus (Pi) concentration (µmol.g^{-1} dry weight (DW)) in *P. vulgaris* leaves that were well-watered (control) and drought-stressed (drought) and cultivated with 80 µM of KH_2PO_4 (Normal P) or supplemented with 200 µM of KH_2PO_4 (High P) for one week before the water withholding treatment. (b) Soil relative water content. (c) Leaf relative water content measured in the 5th trifoliate leaves of control and drought-stressed plants of low or high P conditions. Data are means of three independent experiments. Asterisks indicate statistically significant differences ($p < 0.05$).

Relative soil water content (SWC) was reduced to near 50% in the drought-treated samples, both in the P supplemented and in the lower P pots (Figure 6b), thus demonstrating that drought condition was similar in the two groups of plants. In addition, the relative water content in leaves (RWC) was measured in the four groups of plants. As shown in Figure 6c, leaf's RWC was maintained in the drought-stressed plants, both with and without the addition of P. This result further confirmed that, despite the low SWC, these highly tolerant plants were able to retain their RWC under these stress conditions, as previously observed for this landrace in (Coleto *et al.*, 2014).

To check whether increasing phosphate availability could ameliorate the moderate negative effects of drought in landrace PHA-683, plant biomass was measured in control and 10 days-drought-stressed plants with and without the addition of phosphate supplement (Figure 7).

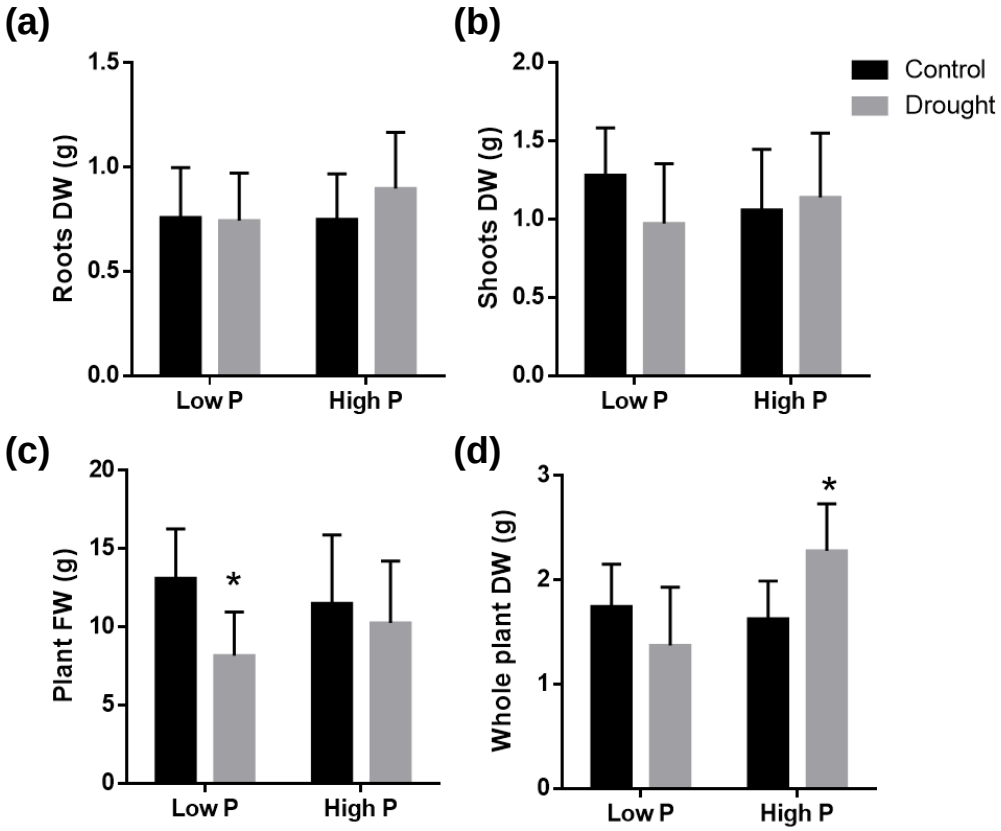

Figure 7. Physiological analysis in plants subjected to drought and cultivated with contribution of P. Effects of 10 days-water-deficit in the biomass of *P. vulgaris* plants that were well-watered (control) or drought-stressed (drought) cultivated with 82 µM KH_2PO_4 (Normal P) or irrigated with 200 µM of KH_2PO_4 (High P) before the drought treatment. (a) Biomass of roots after removal of nodules, (b) shoots biomass, (c) whole plant fresh weight, and (d) whole plant biomass of well-watered (control) and 10 days-drought-stressed (drought) plants. Data are means of three independent experiments with 10 plants from each treatment. Asterisks indicate statistical differences ($p < 0.05$).

Drought did not produce significant changes in root biomass in this landrace, although slightly higher root biomass was found in the drought-stressed high P plants compared to the control or to the normal P plants (Figure 7a). Similarly, drought caused only a slight reduction in the shoot biomass of the lower P plants, although the effect did not reach statistical significance (Figure 7b). As expected, drought reduced the fresh weight of the whole plants. However, the reduction was only significant between the drought and control plants of the lower P nutrition (Figure 7c). Moreover, the whole plant dry weight

of drought-stressed high P plants was significantly higher than in the normal P stressed ones (Figure 7d). These results suggested that drought strongly affected P nutrition and that, at least in part, the moderate negative drought effects on plant biomass of this tolerant common bean landrace could be alleviated when higher P concentration is supplied.

Discussion

Functional genomic tools, such as whole-genome sequencing of transcripts, are currently one of the most useful technologies to clarify the molecular mechanisms of complex traits, such as drought tolerance and, ultimately, to obtain more efficient crops in conditions of abiotic stresses. Transcriptomic analysis, although scarce so far in legumes, has revealed new discoveries associated with the differential expression of genes not easily anticipated with previous physiological studies (Cabeza *et al.*, 2014; Wu *et al.*, 2014). In this work, we did an RNA-seq analysis to dissect the molecular responses to water stress in a common bean landrace, previously characterized as highly tolerant (Coleto *et al.*, 2014). The first surprising result was that, besides the large number of clean reads in each of the sequenced libraries (Supplementary Figure S1), there was only a moderate number of genes that showed differential expression compared to control plants (Figure 1a). As previously shown (Coleto *et al.*, 2014), PHA-683 landrace did not show any physiological symptoms of water stress at 7 days and only moderate symptoms at 14 days of water deprivation. Therefore, only those changes in gene expression related to early or mid-response to stress could be found after the 10 days of water deprivation used in this study. Therefore, the induced and repressed genes found in this study suggested a molecular readjustment in response to the stress in this tolerant plant.

Interestingly, among the genes that change their expression, there were downregulated genes (possibly related to ABA-mediated signaling), such as the PP2C, which is a key negative regulator of ABA signaling (Cutler *et al.*, 2010). Similarly, there was an ABA 8′-hydroxylase gene implicated in ABA catabolism that also appeared repressed by drought (Figure 3). The downregulation of genes putatively forming part of the negative regulators of the core ABA signaling strongly suggested that this tolerant genotype could maintain an efficient ABA response to cope with water stress. Moreover, there were changes in several other possible signaling-related genes, such as genes coding for calcium-binding proteins, membrane receptors, protein kinases, and transcription factors (Supplementary Table S1), thus suggesting that they could be involved in the early or mid-responses to the stress. Among the TFs, we found significant induction of MYB and

WRKY, previously related to ABA-mediated drought responses (Baldoni *et al.*, 2015; Ding *et al.*, 2016; Joshi *et al.*, 2016) and to phosphate deficiency responses (da Silva *et al.*, 2019). Interestingly, WRKY70 has been found to be involved in both brassinosteroids-regulated plant growth and drought responses (Chen *et al.*, 2017) and has been reported as a negative regulator of plant senescence (Besseau *et al.*, 2012). Therefore, WRKY70 could be a key TF whose induction, together with the several DEGs related to stabilization of photosynthetic complexes and membrane and cell wall-associated changes, could be relevant for the high tolerance of this landrace.

Cell wall remodeling under drought stress is a common response in plants (Le Gall *et al.*, 2015; Ezquer *et al.*, 2020). The plant cell wall is a complex structure with critical functions in plant life. The cell wall maintains the structural integrity of the cell by resisting internal hydrostatic pressures while also providing flexibility and supporting cell division and expansion. Many of the genes whose expression changed in response to water-stress in this study were related to the cell wall or extracellular proteins. Drought impacted the water potential of the cell, inducing changes in wall polymer structure and composition, thus justifying the changes in expression of genes coding several expansins, xyloglucan endotransglucosyl hydrolases, extensins, and intrinsic membrane proteins found in the RNA-seq. Due to the high complexity of cell wall and membrane interactions, analysis of these drought-mediated DEGs would require further investigations. In addition, as the cell wall is a strong sink for carbohydrates, it would be interesting to evaluate the relationships among the carbohydrate metabolism-related DEGs found in this study and the cell wall-related changes. Interestingly, several of the genes whose expression changed in the drought treatment code for PHOSPHATE-INDUCED PROTEIN1 (PHI, and PHI-Like) that form part of the large EXORDEUM-like family of genes related with brassinosteroids-mediated cell expansion (Schröder *et al.*, 2009, 2011). Five of these genes were found downregulated in the RNA-seq, according to the negative effect of water stress on cell expansion (Supplementary Table S1).

In addition to the genes coding for Exordeum-like proteins that belong to the phosphate-induced protein 1 (PHI-1), there was a large proportion of the DEGs that were related to phosphorous starvation, thus supporting the relevance of phosphate acquisition for the drought tolerance of this common bean genotype. It has been reported that N_2-fixing legumes require more P than legumes growing on mineral N, and that root nodules are

strong P sinks in legumes. Thus, P concentration in the nodules of soybean (Sa and Israel, 1991) and white lupin (Schulze *et al.*, 2006) from P-deficient plants reach up to 3-fold that of other plant organs. Moreover, P deficiency has a strong detrimental effect on nitrogen fixation in several legumes, including common bean (Hernández *et al.*, 2007). As shown previously (Coleto *et al.*, 2014), nitrogen fixation was only partially inhibited after a severe drought in landrace PHA-863; therefore, a large amount of P was expected to be required to maintain N_2 fixation under these stress conditions. As drought reduced the acquisition of mineral nutrients, including P (Figure 6a), the remobilization of internal P stores, by induction of the several inorganic phosphatases, the lipid phosphatase (LPIN), and the glycerophosphodiester phosphodiesterase found in this work, might help to supply the required P to the N_2 fixing nodules, thus contributing to the tolerance of this genotype. Accordingly, the upregulation by the drought of the expression of the LPIN and phosphodiesterase was abolished in plants growing with a higher amount of P (Figure 4).

However, the supplement of higher P concentration did not reduce the drought-mediated induction of the three phosphoethanolamine/phosphocholine phosphatase/Phospho1 (*PvPhospho*) inorganic pyrophosphatase 1-related genes, indicating that drought was the main factor regulating the expression of these genes. The PHOSPHO1 protein belongs to pyridoxal phosphate PDX family involved in the synthesis of vitamin B6 (pyridoxine and its vitamers) that has been implicated in the defense against cellular oxidative stress caused by abiotic stresses, such as drought, chilling, high light, and ozone (Denslow *et al.*, 2007), and plants with an enhanced level of vitamin B6 have an increased tolerance to oxidative stress and increased resistance to paraquat and photoinhibition (Raschke *et al.*, 2011). Although further experiments will be required to determine whether the tolerant plants accumulate vitamin B6 in response to stress, induction of the Phospho1 or PDX genes found in this study suggested their implication in the protection of the photosynthetic systems under drought stress through the synthesis of vitamin B6, as well as helping to supply P through their phosphatase activity. Moreover, induction of pyridoxal phosphate or other vitamin B6-related compounds, besides acting as a cofactor for many enzymes, is also involved in the synthesis of choline, a precursor in the synthesis of important osmolytes, such as glycine betaine. Interestingly, increasing glycine betaine accumulation has been shown to modulate the phosphate homeostasis in tomato plants (Li *et al.*, 2019).

The effect of phosphate addition was apparent in the group of phosphate-induced genes, PvPHI-1 and PvPHI1-like of the EXORDEUM-like protein family, whose expression levels were reduced under drought in the lower P conditions but not in the P-supplemented ones (Figure 4). As previously mentioned, EXORDEUM proteins are involved in brassinosteroid-mediated cell expansion (Schröder *et al.*, 2011). Interestingly, the supplement of phosphate was shown to alleviate the slight negative effect of drought in the biomass of this tolerant plant (Figure 7). Therefore, it is tentative to speculate that the higher expression level of the EXORDEUM-like coding genes could be related to the higher growth of the P-supplemented plants, even under drought conditions. Nevertheless, although we still do not have a mechanistic explanation on the actual role of regulation of phosphate homeostasis in the tolerance to drought, our results indicated that increasing phosphate availability reduced the negative effect of drought in the biomass of this tolerant plants (Figure 7), thus suggesting that phosphate limitation was among the main constraints caused by drought for the growth of these plants. The regulation of the phosphate nutrition-related genes in response to water deficit was further supported by the presence of several cis-regulatory motives found in their promoter sequences (Figure 5). It would be interesting to study whether the phosphate nutrition-related genes found in this study are also induced in plants fed with nitrate, lacking the strong phosphate sink of the nodules. Similarly, future experiments should be done comparing the induction of these genes in the tolerant and drought-sensitive plants, to ascertain whether the induction of genes involved in the mobilization of phosphorous from cell stores is a factor contributing to the drought tolerance of this genotype. Accordingly, there are reports indicating that selection for drought resistance in common bean also improves yield in phosphorus limited environments (Beebe *et al.*, 2008). Interestingly, P supply has been previously shown to improve legume performances against soil environmental stress factors (Bargaz *et al.*, 2016).

The accumulation of ureides has been considered for years as a symptom of the drought sensitivity of ureidic legumes since these compounds increase particularly in the most sensitive varieties of soybean and common bean (King and Purcell, 2005; Coleto *et al.*, 2014). Synthesis of ureides takes place in the nodules from the oxidation of the *de novo* synthesized purine nucleotides that incorporate the fixed nitrogen (Zrenner and Ashihara, 2006; Coleto *et al.*, 2016). However, in the sensitive common bean plants, drought inhibits nitrogen fixation, and degradation of stored purine nucleotides is the source of

the accumulation of ureides (Alamillo *et al.*, 2010; Díaz-Leal *et al.*, 2012; Coleto *et al.*, 2014). However, drought-stressed tolerant plants of landrace PHA-683 do not accumulate ureides and maintain N_2 fixation under these conditions (Coleto *et al.*, 2014). Accordingly, we did not find any changes in the expression of genes related to ureide synthesis or in the metabolism of purine nucleotides. Interestingly, there was only a reduced number of DEGs related to macromolecules degrading enzymes, such as peptidases, but a total absence of nucleases in the drought-stressed leaves, agreeing to the lack of ureides accumulation in response to stress and the highly tolerant behaviour of this landrace.

In summary, RNA-seq analysis of the drought-tolerant landrace PHA-683 in response to drought revealed responses related with the ABA signaling, including upregulation of several key TF, remodeling of cell walls, synthesis of osmoprotectant oligosaccharides, protection of photosynthetic apparatus, and downregulation of genes involved in cell expansion, but, above all, there was a significant proportion of DEGs related to phosphate starvation response, thus suggesting that acquisition of phosphate could be crucial for the drought tolerance of this common bean landrace. In conclusion, the molecular analysis on a drought-tolerant common bean genotype presented here revealed the importance of phosphorous homeostasis, as well as several other key factors, in response to water stress. These results might be used in the future search for drought-tolerant genotypes or in breeding programs with an aim to obtain highly tolerant common bean plants.

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Supplementary material

Supplementary Table S1. RNA-seq PHA683 results https://www.mdpi.com/2223-7747/9/4/445/s1

Supplementary Table S2. List of Primers

ID gene	Gene name	Primer (5` → 3´)
Phvul.001G021200	*PvPP2C-12*	FW-GGCTTTAGTTGCTGGCTTTG
		Rev-TGCCACTGTTACAACCCAAC
Phvul.002G122200	*ABA8H*	FW-AAGTTCGTGCTCCACAAAGC
		Rev-AAATTGCTTGCGTCCCCAAC
Phvul.008G185700	*WRKY70*	FW-AACGTCTCCACATTGCAGTG
		Rev-TCAGTGGTGCAAGAGACTATGG
Phvul.003G028000	*MYB*	FW-GTTCACAAGCAGCCCCTTTG
		Rev-TGATGAGGTAGCGACTGCAC
Phvul.001G091000	*PvGDP.CDPK*	Fw-TTCTCGGAGGATAATGGTGCTG
		Rev-TTTCCCTCATTCCCATGCTCT
Phvul.002G061900	*PvNRT1*	Fw-TTGAAATTCCCGCAGCATCG
		Rev-TGCCACCGGAACAATGAAAC
Phvul.003G164900	*PvSPX3*	Fw-TTTCCACGGAGAGATGGTCTTG
		Rev-TTCTGAATGAAGGGCAAGCG
Phvul.009G197000	*PvSPX9*	Fw-AGCCAACCAGCACATTGATG
		Rev-CGGTGGCAATGAAAACATGC
Phvul.010G140900	*PvPhospho9*	Fw-CAACTGGGTCGTCGATGAATTG
		Rev-TGTCCATGAGAGTGTTCCAAGG
Phvul.010G141200	*PvPhospho12*	Fw-CGTGGATGAATTGGGTTTCACC
		Rev-TGTGTCCATGAGAGTGTTCCAG
Phvul.010G140800	*PvPhospho8*	Fw-AGATGGCAGTGGAGACTATTGC
		Rev-TATCAAGTCCCACACCGGAAAG
Phvul.001G021400	*PvLPIN*	Fw-TTGTGTCGAGGTCTTTGAGAGG
		Rev-CCACTTCAGCTCCAACAAGTTG
Phvul.008G038300	*PvPHO1-2*	Fw-AGGCCTTGTCATTGTTCTGC
		Rev-AGAGGAGCACAAACGCAATG
Phvul.008G176000	*PvPhi 1*	Fw-TCGGCAAATCACTCACCAAC
		Rev-AACCACGTTGATGGCGTTTC
Phvul.011G004400	*PvPhi 1-L (EXD7)*	Fw-AAGAGGGGCATGTGCAAATG
		Rev-TCGAATCGCTTGGAGGAAGAG
Phvul.009G032100	*PvPhi 1-L (EXD9)*	Fw-ACGGTCCTTTGCTTTATGGC
		Rev-TTTTGGGAGGGTTTGAAGCG
KF033666.1	*PvACT-2*	Fw-GGAGAAGATTTGGCATCACACGTT
		Rev-GTTGGCCTTGGGATTGAGTGGT

Supplementary Table S3. Drought-responsive Cis regulatory motifs found in promoter region of selected phosphorous related gene

GENES	ABRE	ARE	CCAAT-Box	MBSY	MBS	MYB or Myb-bindind site	TC-rich repite	TCA-element	LTR	MYC	CGTCA-motif	TGACG-motif	DRE core	DRE1	TOTAL
PvPhospho8	4	1	1		1	6	1	1							**12**
PvPhospho9		1	1			6			1	4					**11**
PvPhospho12	1	2	1			2			2	2	1	1			**6**
PvSpx3	1	1		1		4				2					**8**
PvSpx9			1			3				1	2	2		1	**5**
PvLPIN		3				9				5	1	1		1	**14**
PvGDP.CDPK	1	1				2	1	1							**3**
PvPhi 1-L (EXD3)	1	5				7			2	2	3	3	1		**10**
PvPhi 1-L (EXD7)	1	2			2	9	1		1	3	1	1			**15**
PvPhi 1-L (EXD9)	2	1			1	11		1			1	1			**14**
PvPHO1-2	7	3				5	1				1	1			**12**
PvPhi 1						6				2					**8**
PvNRT1		1		1		6	1	1	1						**7**

CHAPTER 2

DIFFERENTIAL REGULATION OF DROUGHT RESPONSES IN TWO *PHASEOLUS VULGARIS* GENOTYPES

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Chapter 2. Differential regulation of drought responses in two *Phaseolus vulgaris* genotypes.

Abstract

Drought is probably the most harmful stress affecting common bean crops. Domestication, worldwide spread and local farming practices has entailed the development of a wide variety of common bean genotypes with different degrees of resistance to water stress. In this work, physiological and molecular responses to water stress have been compared in two common bean accessions, PHA-0683 and PMB-0220, previously identified as highly and moderately resistant to water stress, respectively. Our hypothesis was that only quantitative differences in the expression patterns of key genes should be found if molecular mechanisms regulating drought resistance are similar in the two accessions. However, results presented here indicate that the resistance to drought in PMB-0220 and PHA-0683 common bean accessions is regulated by different molecular mechanisms. Differential regulation of ABA synthesis and ABA signaling related genes among the two genotypes, and the control of the drought-induced senescence have a relevant contribution to the higher resistance level of PHA-0683 accession. Our results also suggest that expression patterns of key senescence-related transcription factors could be considered in the screening for drought resistance in common bean germplasm collections.

Introduction

Drought is one of the most harmful abiotic stresses affecting yield of common bean (*Phaseolus vulgaris*) crops, with a great economic and dietary importance, especially in developing countries. Moreover, current climate change and its associated increase of drought episodes are expected to worsen the negative effect of water stress in crops productivity worldwide (Broughton, *et al.*, 2015; Beebe *et al.*, 2013; Long and Ort, 2010). Like other legumes, common bean can use atmospheric nitrogen (N_2) through the symbiotic fixation process (Lugtenberg and Kamilova, 2009), thus allowing a reduced use of fertilizers, promoting a more sustainable agriculture. Typically, the uredic legumes such as common bean (*P. vulgaris*) and soybean (*Glycine max*) transport the symbiotically fixed nitrogen in the form of the ureides, allantoin and allantoate, whereas

most temperate legumes transport their N in the form of amides (Atkins, 1991; Zrenner *et al.*, 2006). Symbiotic nitrogen fixation is rapidly inhibited under conditions of water stress (Sinclair and Serraj, 1995*b*), and the ureidic legumes are in general more susceptible than the amidic ones. Several studies suggested that accumulation of ureides during water stress could be related with the inhibition of symbiotic fixation in ureidic legumes (King and Purcell, 2005). In contrast, subsequent studies have shown that nodule activity in plants subjected to drought is inhibited earlier than the accumulation of ureides in these plants (Alamillo *et al.*, 2010; Gil-Quintana *et al.*, 2013; Coleto *et al.*, 2014). Up to now, there is no a clear relationship between ureides concentration and inhibition of symbiotic fixation during drought, but, on the contrary, also a possible protective role of ureides has been suggested in several plants, probably through their effects regulating abscisic acid (ABA) and jasmonate (JA) levels (Alamillo *et al.*, 2010; Coleto *et al.*, 2014; Watanabe *et al.*, 2014*c*; Takagi *et al.*, 2016).

The phytohormone abscisic acid (ABA) is a key regulator of the plant response to abiotic stressors, but especially to osmotic and water deficit stress. The biosynthesis of this hormone is activated in response to dehydration, through the oxidative cleavage of carotenoids. In contrast, its degradation is induced by rehydration (Roychoudhury *et al.*, 2013), modulating the ABA content according to the environmental conditions. The first steps of its synthesis, from β-carotene to xanthoxin, occur in the plastids, while the final steps take place in the cytosol (Seo *et al.*, 2000*a*,*b*). ABA regulates a plethora of physiological processes, as stomatal closure, growth, as well as dormancy and germination of seeds (Farquhar and Sharkey, 1982; Zhu, 2002; Dong *et al.*, 2015). ABA-mediated responses are also involved in the regulation of other plant developmental processes such as leaf senescence and abscission (Rivero *et al.*, 2007). In response to water deficit, ABA levels rise and initiates the ABA-signaling of these stress responses (Zabadal, 1974). ABA is recognized inside the cells mainly by a family of ABA receptors named Pyrabactin Resistance (PYR)/ PYL(PYR-Like), or Regulatory Component of ABA Receptor (RCAR) (Ma *et al.*, 2009; Park *et al.*, 2010). In the presence of ABA, the ABA receptors PYR/PYL/RCAR bind to, and inactivate type 2C (PP2Cs) Ser/Thr protein phosphatases (Ma *et al.*, 2009; Park *et al.*, 2010; Rodrigues *et al.*, 2013). Inactivation of PP2Cs allows the activation of SnRK2 kinases (Snf1-related protein kinase class 2), which subsequently phosphorylate ABI5/ABFs transcription factors (ABA-

Insensitive5/ABA-responsive element binding factors) which in turn activate the downstream effectors of the stress response (Fujita *et al.*, 2009; Fujii *et al.*, 2010).

Increases in plant resistance to water deficit have been associated to promotion of leaf senescence, in part induced by changes in ABA levels under stress conditions (Rivero *et al.*, 2007). The outcome of leaf senescence is the mobilization of nutrients from senescent to younger tissues, as well as the death of cells with the consequent foliar abscission (Uauy *et al.*, 2006), which, in turn, reduces leaf surface and transpiration rates. It has been suggested that ABA-induced senescence was promoted by the biosynthesis of ethylene (Riov *et al.*, 1990). However, more recent studies showed that, in *Arabidopsis thaliana*, ABA-induced foliar senescence is mediated by an ethylene independent pathway, through direct inhibition of PP2Cs, activation of SnRK2s and activation of the downstream signaling pathway (Zhao *et al.*, 2016). Among the downstream genes involved in activation of senescence and ABA-mediated responses to drought are some of the WRKY transcription factors (TF) (Zou *et al.*, 2004; Liu *et al.*, 2016). WRKY proteins constitute large families that have been classified into three groups depending on its characteristic WRKY domains and zinc finger motifs (Chen *et al.*, 2012). The number of WRKY proteins varies in each species, with 74 and 88 WRKY genes identified in *Arabidopsis* and *P. vulgaris*, respectively (Ülker and Somssich, 2004; Wu *et al.*, 2017), while up to 197 WRKY proteins have been found in *G. max* (soybean) (Schmutz *et al.*, 2010). Although WRKY TFs have been extensively studied in a wide variety of plants, their specific roles in the regulation of drought resistance and senescence in legumes is still largely unknown.

Although common bean is susceptible to drought conditions, finding varieties better adapted to environmental stresses will have a major impact on crop yields and human nutrition, particularly in developing countries (Hummel *et al.*, 2018). The domestication processes and local farming practices have led to the development of a wide variety of common bean genotypes with different degrees of resistance to drought stress (Frahm *et al.*, 2004; Beebe *et al.*, 2013). Characterization of a high number of accessions from the common bean Spanish collection revealed superior performance of several accessions under water scarcity conditions. From these the PHA-0683 landrace appeared as a high yield resistant genotype, whereas the commercial breeding line Great Northern PMB-0220 ranked as a slightly less drought-resistant line (Riveiro, 2012).

Although resistance to drought has been evaluated in many collections of bean germplasm, obtaining varieties resistant to water stress while keeping good productivity is a complex task, because resistance to drought is a trait largely affected by environmental interactions with additive and quantitative effects (Terán and Singh, 2002; Muñoz-Perea *et al.*, 2006). Molecular analyses, boosted by the recent release of common bean genomes, are important tools to understand how drought resistance is achieved in these crops. Genome-wide drought responsive genes have been identified by transcriptomic analysis in several legume crops (Chen *et al.*, 2016; Mashaki *et al.*, 2018; Morgil *et al.*, 2019), including common bean (Wu *et al.*, 2014; Gregorio Jorge *et al.*, 2020).

Previous studies from our group showed that PHA-0683 landrace, besides showing a high yield performance, was also able to maintain good rates of N_2 fixation and did not accumulate ureides in response to the drought stress (Coleto *et al.*, 2014). On the other hand, the PMB-0220 line showed a good level of resistance to drought, although lower than that of PHA-0683 (Muñoz-Perea *et al.*, 2006; Riveiro, 2012; Coleto *et al.*, 2014). In addition, a genome wide transcriptomic analysis of differentially expressed genes under drought conditions in the resistant common bean PHA-0683 landrace, identified changes in ABA signaling-related genes and several key transcription factors known to regulate stress and developmental responses (López *et al.*, 2020*c*).

The hypothesis of the actual work was that the different degree in resistance in the two genotypes studied here should be caused by differential regulation at the molecular level. Therefore, only quantitative differences will be expected, if the higher level of resistance in PHA-0683 and the lower in PMB-0220 are governed by similar regulatory mechanism. In contrast, qualitative differences in the expression patterns of key factors would reveal different mechanisms of resistance to stress in these two genotypes. Therefore, the main aim of this work was the comparison of physiological and molecular responses to drought in the common bean PHA-0683 and PMB-0220 accessions.

Material and methods

Growth Conditions and Plant Material

In this study, *P. vulgaris* highly drought resistant landrace PHB-0683, originated in Monçao (Portugal) of the market class Cranberry, and the less resistant commercial cultivar Great Northern “Matterhorn” PMB-0220 (Kelly *et al.*, 1999; Muñoz-Perea *et al.*,

2006; Coleto *et al.*, 2014), were used. Seeds of the two genotypes were sterilized by rinsing with ethanol (100%) for 30 s, followed by incubation in 5% sodium hypochlorite for 5 min, washed 5–6 folds with sterile distilled water and placed on wet sterile paper in Petri's dishes for 72 h to them to germinate. Germinated seeds were sown in pots with a mix of artificial substrate of perlite/vermiculite (1/2 w/w). Each pot contained 3 plants that were inoculated at sowing with a fresh suspension of *Rhizobium leguminosarum* (ISP 14). Plants were watered every third day with the Rigaud and Puppo's nitrogen-free nutrient solution (Rigaud and Puppo, 1975). Plants were grown in a culture chamber with 300 $\mu E \cdot m^{-2} \cdot s^{-1}$ lighting for 16 h at 26°C and 8 h of darkness at 20°C and 70% relative humidity for 28 days. At this time, which coincides with the end of the vegetative stage and beginning of flowering in these two genotypes, the pots were randomly separated into two groups. Nine pots each containing three plants were used for each treatment and genotype. One of the groups was subjected to 10 days of drought by withholding watering and the other was maintained with regular irrigation, as the control one. During the growth of plants, gravimetric determination of soil water content (SWC) was estimated, following the method described in previous works (Coleto *et al.*, 2014). Finally, samples of the fourth trifoliate leaves were collected, frozen with liquid nitrogen, and stored at −80°C for gene expression and biochemical analyses.

Physiological Analysis

Drought effects on the plant biomass of roots and shoots were measured in the controls, well-irrigated, and drought-stressed plants. Whole plants were collected 10 days after the beginning of the treatment and shoots and roots were weighed to obtain their fresh weight (FW). Tissue samples were desiccated at 75°C for 72 h and weighed again to determine the dry weight (DW). Whole plant biomass was estimated as the sum of roots and shoots dry weights.

Additionally, relative leaf water content (RWC) was measured on the third trifoliate leaf as in previous work (Coleto *et al.*, 2014). For this, after leaf sampling collection from the control and treated plants, the third trifoliate leaf from each plant was weighted and its FW was obtained. Then, the leaves were soaked with distilled water, left overnight at 4°C, and its maximum turgor weight (TW) was obtained. Then, the same leaves were dried for 24 h at 75°C to achieve DW and RWC obtained according to the following formula: $RWC\ (\%) = ((FW - DW)/(TW - DW)) \times 100$.

Chlorophyll Determination

Extraction of total chlorophyll from crushed leaf tissues was done following the method described by Lichtenthaler (Lichtenthaler, 1987). Briefly, 1.5 ml of 80% acetone was added to 90 mg of tissue. The mix was vortexed to homogenize it, centrifuged at 4°C 3000 *g* for 10 min and the supernatant was collected in tubes kept out of direct light. This procedure was repeated three more times and the four supernatants were mixed. Afterward, the absorbance of the samples was measured at 663 and 645 nm, and the content of chlorophylls estimated according to the following formulas: Chlorophyll A ($\mu g.ml^{-1} = 12.25\ (Abs_{663}) - 2.79\ (Abs_{646})$) and chlorophyll B ($\mu g.ml^{-1} = 21.5\ (Abs_{646}) - 5.1\ (Abs_{663})$) in leaf tissues was determined. Chlorophyll expressed as mg $Chl.g^{-1}$ FW.

Determination of catalase and superoxide dismutase activities

Catalase activity was measurement by the disappearance of hydrogen peroxide at 240 nm (Konieczny *et al.*, 2014). First, the leaf extracts were obtained by homogenizing pulverized tissue with extraction buffer, which contain 100 mM phosphate buffer (pH 7); 100 mM EDTA and triton X-100 (0.1%) v/v, at a relation 1:3 (w/v). The homogenized extracts were centrifuged at 20,000 *g* for 10 min at 4°C. Then, the supernatants were collected, and low molecular weight compounds were removed by dialysis in a PD Spin Trap G-15 column (GE Healthcare) at 800 *g* for 1 min at 4°C. Catalase activity was determined using 25 µl of crude extract, added to 975 µl of reaction buffer, which was composed by 50 mM phosphate buffer (pH 7) and hydrogen peroxide (0.05%), and the disappearance of hydrogen peroxide was recorded at 240 nm for 200 s. Enzyme activity unit was defined as the amount of enzyme decomposing 1.0 mole of hydrogen peroxide per minute. Calculations used an absorbance coefficient of 431 $M^{-1}.cm^{-1}$. Activity expressed as $U.mg^{-1}$ protein. Total protein content was determined according to Bradford assay (Bradford, 1976).

Superoxide dismutase activity was measured using a commercial kit (Canvax biotech, S.L.). Leaf material was homogenized on ice using chilled extraction buffer (0.1 M tris-HCl (pH 7.4); 0.5% Triton X-100, 5 mM β-ME and 0.1 $mg.ml^{-1}$ PMSF) at a relation 1:3 (w/v). Afterward, the mix was centrifuged at 20,000 *g* for 10 min at 4°C and the supernatant was collected and dialyzed in a PD Spin Trap G-15 column (GE Healthcare) at 800 *g* for 1 min at 4°C. SOD activity assay was carried out by measuring the disappearance of the colored tetrazolium (WST-1) formazan at 450 nm. SOD activity was

estimated by the % inhibition of formazan obtained by reduction of tetrazolium salts. One-unit SOD specific activity was the amount of protein causing a 50% inhibition of formazan per mg protein. Bradford assay was used to determine protein content (Bradford, 1976).

Nucleic acid isolation and quantification

Total RNA purification was extracted using 50–100 mg of frozen leaf tissue. One ml of Trizol (Nzyol Tzytech) was added to this amount of tissue, homogenized by vortex and incubated for 5 min at room temperature. Next, 200 µl of chloroform were added, the mix was homogenized and incubated 8 min at room temperature. After the incubation, samples were centrifuged at 11,000 *g* at 4°C for 10 min, and 400–600 µl of aqueous phase were collected, transferred to new tubes and 0.8 V isopropanol were added. After gentle mixing samples were incubated 5 min at RT and centrifuged at 11,000 *g* for 15 min at 4°C to precipitate the RNA and the resulting aqueous phase was removed. Then, 1 ml of 75% ethanol was added to the RNA pellet and centrifuged 5 min at 7500 *g* at 4°C. After this, the supernatant was carefully removed and the RNA was resuspended in 200 µl of miliQ water to which 132 µl of lithium chloride 8 M were added and incubated at 0°C overnight for a second precipitation of RNA. The RNA samples were centrifuged at 11,000 *g* at 4°C for 20 min. Supernatant was removed and washed with 75% ethanol before their last centrifugation at 7500 *g* at 4°C for 5 min. Afterward, supernatant was removed, RNA pellets let dry for several minutes and resuspended in 40 µl of miliQ water. Quantification and purity of the total RNA samples were measured using a NanoDrop spectrophotometer and by visual observation after agarose gel electrophoresis.

Analysis of gene expression

First, genomic DNA was removed from the RNA samples, treatment with DNase I (New England Biolabs) at 3°C for 10 min. Synthesis of first strand cDNA was done using 2.5 µg of DNase-treated RNA using PrimeScript™ reverse transcriptase (TAKARA) following the manufacturer's instructions. Analysis of gene expression was carried out by qRT-PCR using iQ SYBR-Green supermix (Bio-Rad) with specific primers for each gene (Supplementary Table S2) in an iCycler iQ System (Bio-Rad). The program used was based in an initial denaturation and an activation of a Taq polymerase at 95°C for 5 min followed of 40 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 30 s and, finally, 80 cycles at 60°C for 30 s. Relative expression of each gene for control and drought-stressed

samples was normalized to that of *Actin-2* and estimated according to Livak and Schmittgen, 2001.

Analysis of promotor regulatory motives in wrky coding genes

1.5 Kb DNA sequences of the 5′ upstream regions of each gene were obtained from Phytozome database v12 (https://phytozome.jgi.doe.gov/) (Schmutz *et al.*, 2014), and search for regulatory cis-elements was done using plant CARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot *et al.*, 2002).

Experimental design and statistical analysis of data

At least three independent samples were analyzed in this research for each condition (Control and drought) and genotype (PHB-0683 and PHA-0246). Each sample consisted in three pots per condition, each containing three plants. Statistical analysis was done by Student's *t-test* and ANOVA using GraphPad Prism 6 software package (https://www.graphpad.com/).

Results

To determine if the different degrees of resistance to water stress in two common bean genotypes are indeed mediated by differential molecular responses, the highly tolerant PHA-0683 landrace and the PMB-0220 breeding line (Riveiro, 2012; Coleto *et al.*, 2014) were subjected to 10 days of water deficit and the responses were compared to those of the well-irrigated, control plants, from each genotype.

Physiological effects of drought in two common bean drought tolerant plants

Drought effects on the relative water content of soil and leaves, and in the plant biomass were compared between the control plants and the plants subjected to 10 days of water deficit on the PHA-0683 landrace and the PMB-0220 breeding line.

The results in Figure 1a show that the soil moisture (relative soil water content) was above 80% of soil water capacity (field capacity) in the control group of both cultivars, whereas, after 10 days of drought treatment, soil water content decreased up to 40% in the PMB-0220 line and up to 50% SWC in the PHA-0683, thus suggesting that PHA-0683 plants used less water from soil than PMB-0220 ones. However, despite the shortage in available soil water content, there were no significant differences in the relative leaf water content (LWC) between control and drought conditions in any of the two cultivars (Figure 1b).

Nevertheless, while PHA-0683 did not show any reduction in leaf water content after the water stress, a slight decrease in LWC was found in leaves of PMB-0220, agreeing with the degree of drought resistance in these plants.

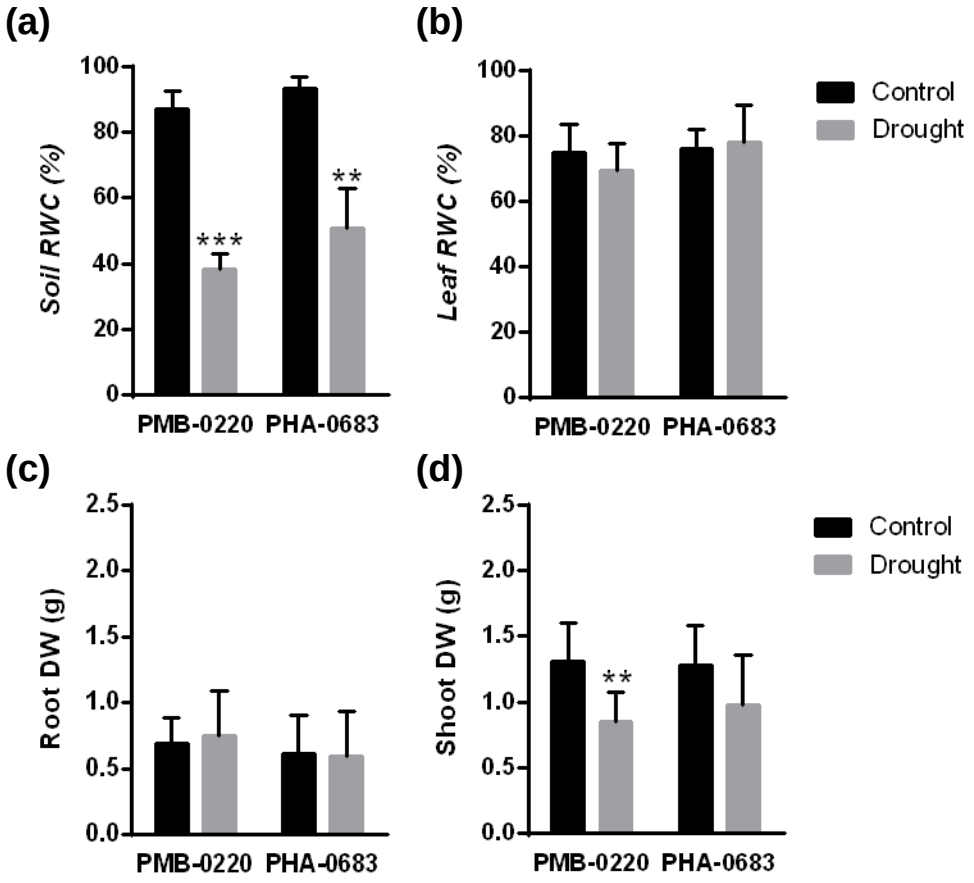

Figure 1. Physiological analysis from common bean PMB-0220 and PHA-0683 genotypes. Physiological parameters of control and 10 days water deficit stressed plants of common bean PMB-0220 breeding line and PHA-0683 landrace, grown under symbiotic nitrogen fixation conditions for 28 days before the stress treatment. Relative water content of (a) soil and (b) leaf, and biomass of (c) roots and (d) shoots. Data are means of three independent experiments and asterisks indicate statistically significant differences (** $p < 0.005$) and (*** $p < 0.0005$).

Determination of the effect of water deprivation in the biomass of roots and shoots showed that, although there were no significant differences in the total roots biomass between control and drought conditions in any accession, a slight increase in the biomass of roots, with respect to the control conditions, was observed in PMB-0220 plants (Figure 1c). In contrast, a significant reduction in shoot biomass was found in the water-stressed PMB-220 plants, compared to their control condition (Figure 1d). On the contrary, there were no significant differences between control and drought conditions in the aboveground biomass in PHA-0683 plants (Figure 1d). Moreover, under control

conditions, similar biomass was found in the roots and shoots of the two genotypes, indicating that the moderate differences in water use and in shoot biomass were not caused by phenological or developmental differences of the plants.

Changes in total chlorophyll contents in response to drought

Drought stress is known to trigger senescence symptoms leading to a reduction of chlorophyll contents in the leaves of stressed plants. To determine whether there where changes in the chlorophylls levels in response to the stress in the PMB-0220 and PMB-0683 genotypes, contents of both, chlorophylls a and b, were measured in all the developed leaves present in plants submitted to 10 days of drought treatment and in control, well-irrigated plants (Table 1). The results, summarized in the Figure 2a, showed that the oldest leaves in the drought stressed PMB-0220 plants had lower chlorophyll content than control ones. Moreover, although chlorophylls a and b were reduced in the second trifoliate leaves of PMB-0220 after 10 days of water stress, the reduction was higher in chlorophyll a than chlorophyll b (Table 1). Instead, the leaves of PHA-0683 drought stressed plants did not show any significant reduction in chlorophylls content with respect to its control conditions (Figure 2b). On the contrary, chlorophyll levels appeared to be slightly higher in the youngest leaves of the water-stressed PHA-0683 plants, probably reflecting the slight reduction of the area of the youngest leaves, that accounts also for the moderate decrease in shoot biomass in these plants.

Table 1. Concentration of chlorophyll a, b and total chlorophyll in the 2nd, 3rd, 4th and 5th trifoliate leaves of *P. vulgaris* PMB-0220 and PHA-0683 subjected to well-watered and water deficit conditions for 10 days.

		[Chlorophyll a]		[Chlorophyll b]		[Chlorophyll a+b]	
		PMB-0220	PHA-0683	PMB-0220	PMB-0683	PMB-0220	PHA-0683
2nd TFL	Control	1.270 ± 0.209	1.475 ± 0.279	0.634 ± 0.115	0.732 ± 0.151	2.013 ± 0.336	2.362 ± 0.417
	Drought	0.501 ± 0.144	1.485 ± 0.201	0.429 ± 0.207	0.634 ± 0.070	1.078 ± 0.451	2.329 ± 0.294
3rd TFL	Control	1.664 ± 0.225	1.673 ± 0.160	0.789 ± 0.146	0.873 ± 0.148	2.880 ± 0.085	2.795 ± 0.317
	Drought	1.452 ± 0.195	1.589 ± 0.186	0.704 ± 0.071	0.729 ± 0.044	2.246 ± 0.140	2.546 ± 0.230
4th TFL	Control	1.599 ± 0.215	1.669 ± 0.172	0.891 ± 0.082	0.877 ± 0.125	2.867 ± 0.142	2.798 ± 0.267
	Drought	1.546 ± 0.251	1.657 ± 0.148	0.778 ± 0.118	0.906 ± 0.148	2.551 ± 0.294	2.813 ± 0.267
5th TFL	Control	1.587 ± 0.178	1.797 ± 0.174	0.687 ± 0.156	0.886 ± 0.125	2.685 ± 0.166	2.950 ± 0.251
	Drought	1.628 ± 0.134	1.776 ± 0.160	0.779 ± 0.191	0.889 ± 0.297	2.644 ± 0.206	2.926 ± 0.487

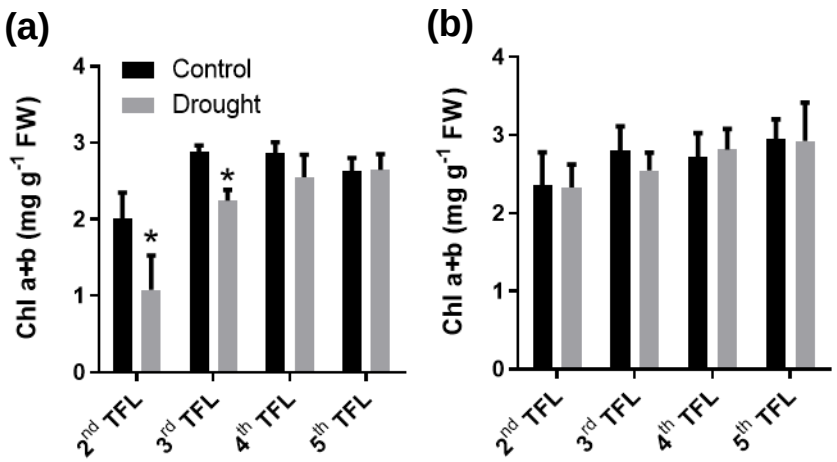

Figure 2. Concentration of chlorophylls in the 2nd, 3rd, 4th, and 5th trifoliate leaves of *P. vulgaris*. Concentration of chlorophylls in PMB-0220 (a) and PHA-0683 (b) plants under control conditions and after 10 days of drought. Data were means of three independent repetitions, and 3 plants were used per sample in each replicate. Asterisks indicate statistically significant differences *(* $p < 0.05$)*

Analysis of genes expression related to ABA-mediated response

In a previous study, several genes of the core ABA signaling were found to change in response to drought in the highly resistant PHA-0683 landrace (López *et al.*, 2020*c*). To elucidate whether similar ABA-mediated responses take place in the PMB-0220 line, levels of relative expression of key genes for ABA synthesis (9-Cisepoxycarotenoid Dioxygenase, NCED), degradation ((+)-Abscisic acid 8'-Hydroxylase, ABA 8'H), ABA signaling (type 2C protein phosphatases, PP2Cs) and downstream responsive genes, as the proline synthesis (Δ1-Pyrroline–5-Carboxylate Synthase, P5CS), were measured in samples of the two genotypes. Expression patterns of these ABA-related genes were compared in leaf tissues from control and from plants subjected to 10 days of drought from each genotype (Figure 3a–d). As shown in Figure 3a–d, the relative expression of *PvNCED3*, *PvABA8'H* and *PvP5CS10*, coding for ABA synthesis, ABA degradation and ABA-responsive genes, respectively, was significantly induced by drought in the PMB-0220 line. In contrast, the expression of these genes was repressed by water deprivation in PHA-0683. On the other hand, *PvPP2C.12*, coding for a phosphatase 2C involved in repression of ABA-responses (Bai *et al.*, 2019), showed a reduction in its levels under drought conditions in both cultivars (Figure 3c), although *PvPP2C.12* gene expression was only highly repressed under drought conditions in PHA-0683, with only a slight, no significant decrease of expression levels in PMB-0220 samples (Figure 3c).

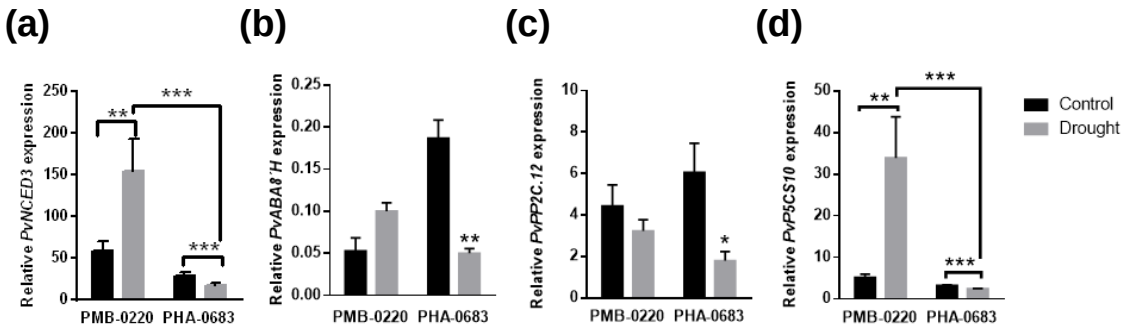

Figure 3. Relative expression of abscisic acid (ABA)-related genes in leaves from control and 10 days-drought-stressed plants of PMB-0220 and PHA-0683 lines, grown under symbiotic nitrogen fixation conditions. Relative expression of (a) *PvNCED3*; (b) *PvABA8'H*; (c) *PvPP2C.12* and (d) *PvP5CS10* in response to drought. Data were means of three independent samples and asterisks indicate statistically significant differences (* $p < 0.05$) (** $p < 0.005$) and (*** $p < 0.0005$).

Analysis of genes expression related to senescence in response to drought

To investigate if the differences in the ABA-related genes among the two genotypes could also be observed in transcription factors involved in key processes affecting drought resistance in the PMB-0220 and PHA-0683 lines, the expression of several genes related to the activation or inhibition of senescence, and whose expression could be mediated by ABA levels, was measured in leaf samples from control and drought stressed plants from the two genotypes. The results in the Figure 4a–c show that the drought-mediated changes in the expression of three genes encoding WRKY TF exhibited significant differences between the PMB-0220 and PHA-0683 plants in response to the stress. Expression of *PvWRKY53* gene, directly related with induction of senescence (Miao *et al.*, 2004; Besseau *et al.*, 2012), showed a significant induction after 10 days of drought with respect to its well-irrigated control in the PMB-0220 line (Figure 4a). On the contrary, there were no significant differences in expression of *PvWRKY53* between the control and stressed leaf tissues in the PHA-0683 plants (Figure 4a). However, *PvWRKY53* showed a higher expression level under control conditions in PHA-0683 than in PMB-0220. Moreover, the expression patterns of *PvWRKY57*, also related to induction of senescence and drought resistance (Abe *et al.*, 1997), were similar to these of *PvWRKY53* in response to drought. *PvWRKY57* was significantly overexpressed at 10 days of water stress compared to the control conditions in the PMB-0220 cultivar, while in PHA-0683 there were no significant differences between control and water stressed samples, although, in this landrace, *PvWRKY57* was overexpressed with respect PMB-0220 (Figure 4b). In

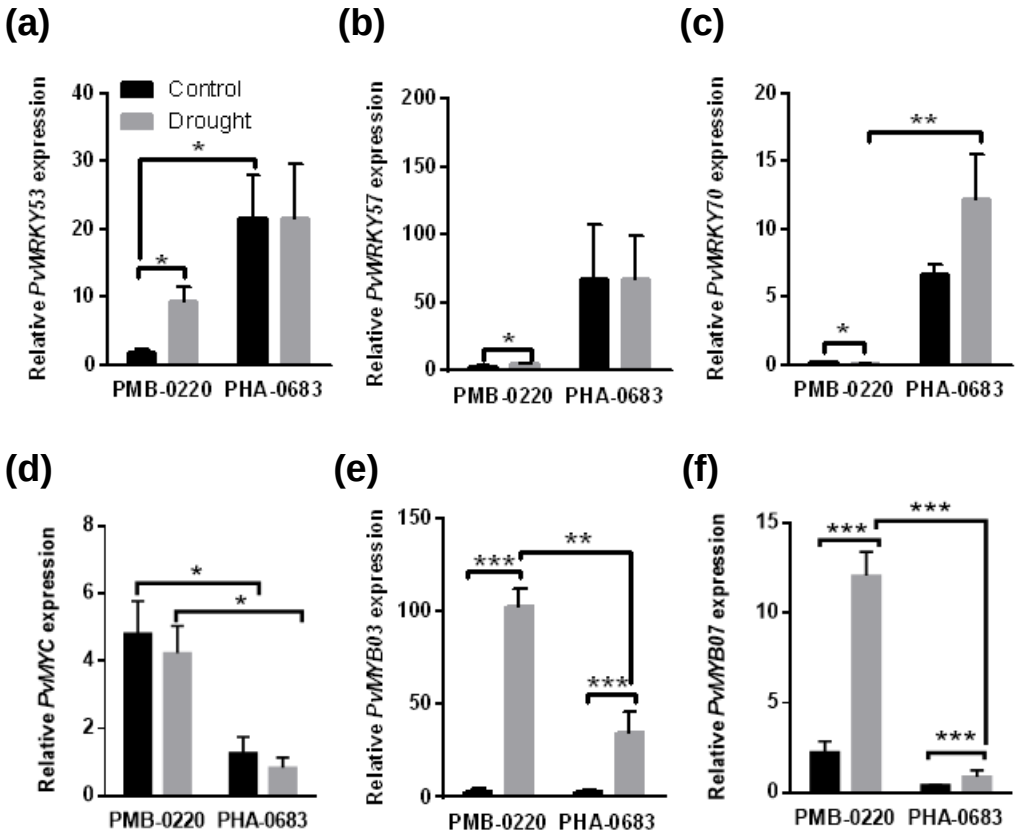

Figure 4. Relative expression of key transcription factors related with senescence and drought responses in leaves from control and 10 days-drought-stressed PMB-0220 and PHA-0683 plants, grown under symbiotic nitrogen fixation conditions. Relative expression of (a) *PvWRKY53*; (b) *PvWRKY57*; (c) *PvWRKY70*; (d) *PvMYC*; (e) *PvMYB03*; and (f) *PvMYB07* in response to drought. Data are means of three independent experiments and asterisks indicate statistically significant differences (* $p < 0.05$) (** $p < 0.005$) and (*** $p < 0.0005$).

addition, the *PvWRKY70* gene, which has been related with inhibition of senescence (Zhou *et al.*, 2008), showed an opposite expression pattern in one cultivar or the other during drought conditions. *PvWRKY70* was significantly downregulated under drought conditions in PMB-0220, whereas it showed an induction of its expression at 10 days of water stress in leaves from PHA-0683 plants. Finally, the results presented in Figure 4d–f show that the expression of genes coding for MYC and MYB transcription factors involved in the responses to abiotic stress was also different among the two common bean genotypes. *PvMYC* was slightly downregulated under drought conditions in both cultivars, but its expression was significantly higher in the control and drought conditions in PMB-0220 than in PHA-0683. In contrast, two MYB genes, *PvMYB03* and *PvMYB07*, showed a significant induction of their expression after 10 days of drought in each

genotype, although the level of induction was also higher in the common bean PMB-0220 than in the PHA-0683.

Discussion

The search for drought resistant common bean varieties has increased due to the worsening of drought episodes by the current climate change conditions (Hummel *et al.*, 2018). Most research until now has focused on the search for the most productive genotypes, based on the physiological and agronomical characterization of cultivars or landraces that evolved under such unfavourable conditions, or that have been obtained in breeding programs (Muñoz-Perea *et al.*, 2006; Riveiro, 2012). These studies have revealed that there are large phenotypic differences in the drought tolerance in common bean genotypes (Beebe *et al.*, 2013). However, despite the large effort posed in the search for drought-resistant common bean lines, studies aimed to dissect the molecular mechanism that govern these phenotypic differences are scarce till now. In this work, molecular and physiological responses of two common bean genotypes have been compared. From these, landrace PHA-0683 ranked as a highly drought-resistant genotype, whereas PMB-0220 was a commercial high yielding line, that ranked as moderately resistant to drought conditions (Riveiro, 2012). In a recent genome wide transcriptomic analysis in leaf tissue of PHA-0683 landrace, differential expression of key genes related with ABA-mediated responses and the regulation of senescence by drought was observed (López *et al.*, 2020*c*).

The results in physiological parameters shown in Figure 1 revealed that there were no significant differences in the leaf relative water content and biomass accumulation between the control and drought-stressed plants of PHA-0683 landrace, with only a slight reduction in the shoot biomass after 10 days of water stress (Figure 1d), which correlated to a discrete reduction in the number of pods and seeds caused by water deficit in field experiments (Riveiro, 2012). However, common bean PMB-0220 breeding line showed a significant decrease in shoot biomass and a slight increase in root biomass under drought conditions against control conditions (Figure 1c, d). This slight root growth is a common response related to the need to reach soil layers with higher moisture levels (Singh, 2007). The decrease in aerial dry matter in both cultivars also corresponded to a general slow-down of cell expansion, to reduce the gas exchange surface and the loss of water in the leaves in response to water scarcity. Moreover, the smaller effects observed in the drought-stressed PHA-683 plants agrees with the higher level of drought resistance

reported for this landrace in comparison with the PMB-220 breeding line. Noteworthy, most of the physiological and phenological parameters related to growth habits, days to flowering, shoot and root biomass are similar in the two common bean genotypes (Riveiro, 2012). Therefore, the small increase in root and decrease in shoot biomass in PMB-0220, but not in PHA-0683, should be explained as a differential response to drought, in turn, caused by metabolic and molecular changes in the two lines. Moreover, the higher relative soil water content after the water deficit treatment in the PHA-0683 landrace strongly suggests that this genotype is better adapted to restrict water loss, probably through a faster ABA-mediated stomatal closure (Farquhar and Sharkey, 1982; Lizana *et al.*, 2006).

In a previous study, the transcriptome changes in response to drought showed down-regulation of genes coding for proteins that are repressors of ABA signaling, as the PP2Cs, and of ABA degradation, as ABA 8′ hydroxylase, suggesting that ABA-mediated responses were activated in PHA-0683 landrace (López *et al.*, 2020*c*). In this work we have compared the expression levels of genes coding for key enzymes in ABA synthesis, as the 9-cis-epoxycarotenoid dioxygenase (NCED) (Tuteja, 2007), signaling as phosphatase PP2C, degradation as ABA 8′ hydroxylase, and ABA-responsible genes, like the one coding P5CS, involved in the synthesis of proline.

Abscisic acid is synthesized *de novo* in response to dehydration, through transcriptional regulation of ABA biosynthetic genes (Iuchi *et al.*, 2001). Several studies have shown that NCED was the key enzyme involved in ABA biosynthesis (Qin and Zeevaart, 1999; Seo and Koshiba, 2002). Nine NCED genes have been identified in *Arabidopsis thaliana*, of which NCED3 (*AtNCED3*) controlled the level of endogenous ABA under drought stress conditions (Iuchi *et al.*, 2001). In *P. vulgaris* there are three NCED-related genes, from which *Phvul.005G051600* showed the highest homology to NCED3 from *Arabidopsis* and was the one chosen in this analysis.

In PMB-0220 plants, *PvNCED3* showed a significantly higher level of expression in drought-stressed than in control leaves (Figure 3a), suggesting induction of the synthesis of ABA during water deficit conditions. In contrast, the expression level of *PvNCED3* was lower under drought conditions with respect to the control in PHA-0683 landrace, and it was also lower than in water-stressed samples from the PMB-0220 genotype (Figure 3a). In contrast, the *PvABA8'H* and *PvPP2C.12* genes were down-regulated by water stress in PHA-0683, with respect to control conditions, while in PMB-0220, these

genes were either up-regulated, or remained unchanged in response to stress (Figure 3b, c). *PvPP2C.12* gene encodes a PP2C phosphatase recognized as a negative regulator of ABA-mediated response (Cutler *et al.*, 2010), and *PvABA8'H* encodes the ABA-8'-hydroxylase protein that catalyzes the key oxidation step in the ABA catabolism pathway (Krochko *et al.*, 1998; Nambara and Marion-Poll, 2005). Therefore, expression patterns of these ABA-related genes strongly suggest different regulatory mechanism in the two cultivars. Thus, while water stress seems to promote the ABA biosynthesis in PMB-0220 cultivar, it reinforces the ABA-mediated responses in the PHA-0683 landrace by repressing negative regulators and inhibiting its degradation. Nevertheless, although 10 days of treatment were chosen because PHA-0683 did not show any appreciable symptoms of stress at earlier times (Coleto *et al.*, 2014), we cannot exclude that different kinetics could be responsible for these results; thus, further analysis should be done to determine whether ABA synthesis could be induced earlier in these plants.

Apart from the ABA-mediated regulation of stomatal conductance, triggering stomatal closure, ABA also promotes the synthesis of compatible metabolites such as proline, which acts as active compounds to allow osmotic adjustment during water stress (Roychoudhury *et al.*, 2015). Increases in proline concentration is a widespread response to drought, cold, and salt stress, often regarded as a basic strategy for the protection and survival of plants under abiotic stress. Proline accumulation is mediated by the induction of the P5CS gene, encoding a Δ1-pyrroline-5-carboxylate synthase, which catalyzes the first and rate-limiting step in the synthesis of this amino acid.

In most plants, there are two isoforms of P5CS that differ in their temporal and spatial expression patterns (Székely *et al.*, 2008; Kirchberger *et al.*, 2008; Szabados and Savouré, 2010). In common bean, there are four genes coding P5CS proteins, from which *PvP5CS10* (*Phvul.010G015400*) showed the highest homology to P5CS1, responsible for the accumulation of proline under drought stress in *Arabidopsis*. The relative expression of *PvP5CS10* increased significantly after 10 days of drought in PMB-0220 (Figure 3d), suggesting that synthesis of proline might contribute to the osmotic adjustment in these plants that showed no significant differences in the leaf RWC under water stress with respect to the control (Figure 1b). In contrast, the proline synthesis gene, *PvP5CS10*, was repressed compared to control conditions in the PHA-0683 plants, despite that RWC was also maintained in leaves subjected to 10 days of drought in these plants. Interestingly, genes coding for glycine-betaine were found as induced by water stress in the

transcriptome analysis of this landrace (López *et al.*, 2020*c*), suggesting different osmotic adjustment mechanisms in the two genotypes. Moreover, a recent study demonstrated that concentration of proline does strongly correlate with the susceptibility to drought in common bean landraces and accessions (Székely *et al.*, 2008), thus also agreeing with the lower resistance of the commercial cultivar PMB-0220 compared to the PHA-0683 landrace.

Besides acting as a compatible osmolyte, proline produced under stressful conditions also acts as a free radical scavenger and an activator of ROS detoxification pathways. Moreover, the accumulation of proline can function as a signaling molecule, inducing the expression of stress-responsive genes, including those encoding enzymes that scavenge ROS (Hossain *et al.*, 1996). Accordingly, preliminary results from our group indicate that induction of some antioxidant activities, like superoxide dismutase (SOD) and catalase, were higher in PMB-0220 plants than in the PHA-0683 ones (Supplementary Figure S1)

In addition to osmotic regulation, acceleration of leaf senescence is a common response of many plants to adapt to adverse conditions (Zhao *et al.*, 2016). Leaf senescence is a developmental process regulated via changes in hormonal balance, especially associated with the decrease in cytokinin (CKs) and increase in ABA contents, although levels of jasmonic acid (JA), ethylene, and salicylic acid are also important (Gan and Amasino, 1995; Yang *et al.*, 2003). These changes in hormonal balance are transduced by transcription factors including several members of the WRKY, NAC, MYC, and MYB TF families. We found that the expression of *PvWRKY53*, a key gene related to the induction of senescence in *Arabidopsis* (Miao *et al.*, 2004), had a significant induction in leaves of PMB-0220 plants subjected to 10 days of water stress, while there were no significant differences among drought and control samples from PHA-0683 plants (Figure 4a). On the contrary, *PvWRKY70*, which is considered as a senescence repressor gene in *Arabidopsis* (Besseau *et al.*, 2012), was significantly upregulated in the foliar tissue of plants subjected to drought in PHA-0683 landrace (Figure 4c), confirming the results from the transcriptome study of these plants (López *et al.*, 2020*c*). Conversely, *PvWRKY70* gene was downregulated in leaves from the PMB-0220 line subjected to drought. Besides being involved in senescence regulation, some genes coding for WRKY TFs are also directly related to the tolerance to water stress. *PvWRKY53* and *PvWRKY57* might be part of this group, as reported for their closest homologs in soybean (*GmWRKY54*) and *Arabidopsis* (*AtWRKY57*) (Zhou *et al.*, 2008; Jiang *et al.*, 2012). The

expression patterns of these TF, shown in Figure 4, strongly agree with the high tolerance of the common bean PHA-0683 landrace, where, in addition to the *PvWRKY70* inhibition of foliar (Figure 4c), high levels of *PvWRKY53* and *PvWRKY57*, found already under control conditions, may promote the protection against water stress. Moreover, the expression of WRKY genes was always higher in samples from PHA-0683 than in the those from the PMB-0220 cultivar (Figure 4a, b), thus confirming the differential behaviour of these regulatory genes in the two genotypes.

Furthermore, MYB- and MYC-type transcription factors have been related with ABA and jasmonate-mediated responses that regulate tolerance to abiotic stresses as drought (Abe *et al.*, 1997; Kazan and Manners, 2013). MYB transcription factors have also been associated with the regulation of senescence (Guo and Gan, 2011). In this work, we investigated the expression of two MYB and one MYC encoding genes, whose expression was previously found to change in response to drought in PHA-0683 landrace (López *et al.*, 2020*c*). Two genes that code for MYB TFs (PvMYB03 and PvMYB07) were overexpressed in PMB-0220 and PHA-0683 after 10 days of drought (Figure 4e, f), although the induction was higher in PMB-0220 than in the PHA-0683 genotype, agreeing with the induction of ABA synthesis genes in PMB-0220 plants. In contrast, the MYC-coding gene showed no significant expression changes in any of the two lines, but, again, its expression levels were higher in the leaves from PMB-0220 than in those from PHA-0683 plants.

Analysis of the promoter regions of *PvWRKY53, PvWRKY57, and PvWRKY70* genes revealed that they contain several cis-acting elements related to the binding of MYC and MYB TF. Moreover, the promoters of these WRKY genes also contain other stress-related cis-acting elements, such as ABRE (ABA responsive element), TCA-element (salicylic acid responsive element), CGTCA/TGACG-motif (MeJa responsive element), P-box (gibberellin-responsive element), and TGA-element (auxin-responsive element) (Supplementary Table S1). The presence of several abiotic stress-related motives in the WRKY genes suggests that, indeed, they would play a prominent role in drought resistance in the two common bean cultivars. On the other hand, WRKY TFs regulate the expression of downstream genes involved in general features of senescence, as remobilization processes and induction of antioxidant enzymes. In fact, direct interaction of WRKY53 with the promoter of catalase genes has been reported (Zentgraf and Doll, 2019). Accordingly, we found induction of catalase activity only in drought-stressed

leaves from PMB-0220, agreeing with the induction of *PvWRKY53* expression in these plants, but not in the PHA-0683 ones.

Chlorophyll degradation is among the early symptoms of leaf senescence (Hörtensteiner, 2006; Lim *et al.*, 2007). Determination of chlorophyll concentration in the two common bean genotypes revealed that chlorophyll degradation occurred earlier in the PMB-0220 than in the PHA-0683 plants submitted to water stress (Table 1; Figure 2). These results coincided with the more pronounced decrease in the aerial biomass under water stress in PMB-0220 plants and with the expression patterns of *PvWRKY53* and *PvWRKY70*, found in these two genotypes.

Regulation of senescence under drought conditions may have both beneficial and detrimental effects and there are studies showing that the delay of foliar senescence is related to resistance to water deficit (Rivero *et al.*, 2007). Contrary, there are many other reports highlighting that the increase in ABA levels directly promotes senescence, with the consequent remobilization of nutrients from senescing organs to the youngest tissues (Volaire and Norton, 2006; Christ and Hörtensteiner, 2014; Zhao *et al.*, 2016). Inhibition of leaf senescence in PHA-0683 plants under drought is also consistent with a lower induction of NCED gene controlling ABA synthesis, compared to the results in PMB-0220 accession (Figure 3a). This also agrees with previously reported results showing that PHA-0683 plants are able to maintain symbiotic nitrogen fixation, reducing the need for nutrient remobilization (Coleto *et al.*, 2014). In contrast, the promotion of senescence in the oldest leaves of PMB-0220 plants might help to acquire the required nitrogen to nourish young tissues under the water stress, thus also contributing to the drought resistance of these plants. Although further data are required to ascertain whether control of senescence is a unique adaptation of PHA-0683 landrace, or, instead, it could have an important role in the resistance of other common bean genotypes. Moreover, our results highlight the need to implement more broad-range molecular analysis, including whole genome sequencing and transcript abundance under environmentally stressful conditions in different bean genotypes, that will help to dissect drought resistance regulation in this important crop.

In summary, we show that the drought tolerance in PMB-0220 and PHA-0683 common bean accessions is regulated by different molecular mechanisms and that control of the drought-induced senescence seems to be relevant for the better performance of PHA-0683 accession under drought conditions. Our results also suggest that expression patterns of

key TF as WRKY53 and WRKY70, or the proline synthesis P5CS, could be used for easy screening of drought resistance/susceptibility in common bean germplasm collections.

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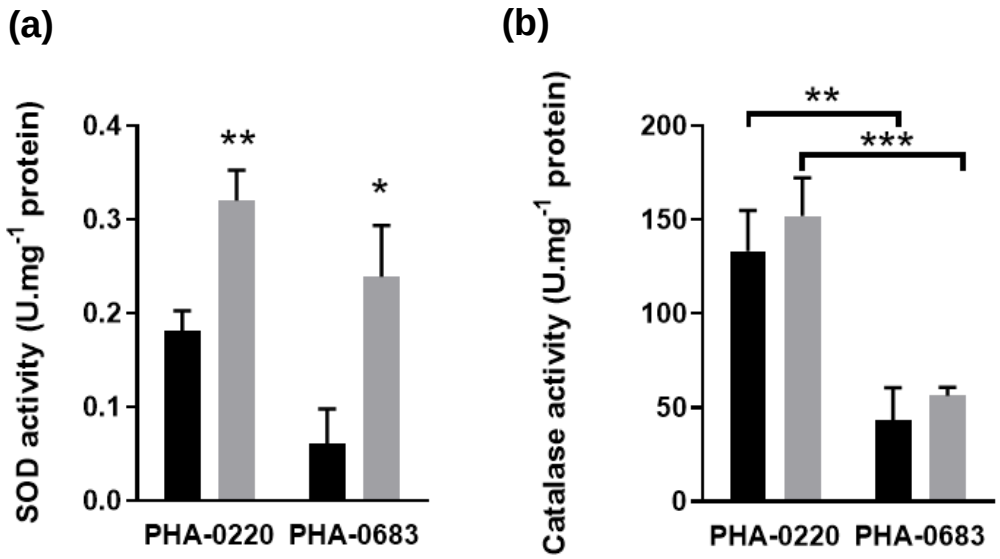
Supplementary material



Supplementary Figure S1. Antioxidant activities Superoxide dismutase (SOD) (a) and catalase (b) enzymatic activity in leaves from PMB-0220 and PHA-0638 common bean from control and 10 days water-stressed plants. (a) SOD activity estimated by the % inhibition of formazan dye formed by reduction of tetrazolium salts. (b) Catalase activity was determined by measuring the disappearance of hydrogen peroxide at 240 nm Measurements were done in three independent biological samples and two measurements were done for each sample.

Supplementary table 1. Cis regulatory motifs found in the promoter regions of selected senescence related WRKY genes

Motives	Motives sequences and responsivenes	*PvWRKY53*	*PvWRKY57*	*PvWRKY70*
ABRE	ACGTG (ABRE) / TACGTG (ABRE3a) / CACGTA (ABRE4)	2	0	3
ARE	AAACCA	3	1	3
MYB	TAACCA / CAACAG / TAACTG (MYB) /AACCTAA (MRE)	14	4	4
MYC	CATTTG	5	2	2
CGTCA-motif	CGTCA MeJA responsive motif	1	4	0
TGACG-motif	TGACG MeJA responsive motif	1	4	0
TC-rich	TC-rich element, defense and stress	0	1	0
TCA-element	TCA-element, salicylic acid	0	0	1
TGA-element	TGA-element, auxin	0	0	1
Total		**26**	**16**	**14**

Supplementary Table S2. List of primers used in this study.

ID gene	Gene name	Primer (5` → 3´)
Phvul.005G051600	*PvNCED3*	Fw- TTAAGGACCTGGCTTCCACATC
		Rev- GTGATGGTGTTGGTGTTGGAAG
Phvul.003G278400	*Pv8ABAH*	Fw- AAGTTCGTGCTCCACAAAGC
		Rev- AAATTGCTTGCGTCCCCAAC
Phvul.001G021200	*PvPP2C-12*	Fw- GGCTTTAGTTGCTGGCTTTG
		Rev- TGCCACTGTTACAACCCAAC
Phvul.010G015400	*PvP5CS10*	Fw- GATGTTGAGGGCCTTTACAGTG
		Rev- CTGCCCAATCTTGACTTGTCTC
Phvul.002G297100	*PvWRKY53*	Fw- ACGCCCAAATGGATGGATCATG
		Rev- TGGCTCCGAGGATGTCTTTTTG
Phvul.002G081600	*PvWRKY57*	Fw- CAGCAAATGCACGGTGAAGAAG
		Rev- AACGGTATGATGGCAGTGCTG
Phvul.008G185700	*PvWRKY70*	Fw- AACGTCTCCACATTGCAGTG
		Rev- TCAGTGGTGCAAGAGACTATGG
Phvul.003G285700	*PvMYC2*	Fw- GAAAGGGGAATTGGAGAAGC
		Rev- ATCAGCTTGCTCGTCGTTTC
Phvul.003G028000	*PvMYB03*	Fw- GTTCACAAGCAGCCCCTTTG
		Rev- TGATGAGGTAGCGACTGCAC
Phvul.007G224600	*PvMYB07*	Fw- TGATGATGCAGCAACTGAGTGC
		Rev- TATTTGCTGCTCCTCCAACTGC
KF033666.1	*PvACT-2*	Fw-GGAGAAGATTTGGCATCACACGTT
		Rev-GTTGGCCTTGGGATTGAGTGGT

CHAPTER 3

TRANSCRIPTOMIC AND METABOLOMIC ANALYSIS REVEALS THAT SYMBIOTIC NITROGEN FIXATION ENHANCES DROUGHT RESISTANCE IN COMMON BEAN

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Chapter 3. Transcriptomic and metabolomic analysis reveals that symbiotic nitrogen fixation enhances drought resistance in common bean

Abstract

Common bean (*Phaseolus vulgaris L.*), one of the most important legume crops, use atmospheric nitrogen through symbiosis with soil rhizobia reducing the nitrogen fertilization needs. However, this legume is particularly sensitive to drought conditions, prevalent in arid regions where this crop is cultured. Therefore, studying the response to drought is important to sustain crop productivity. We have used integrated transcriptomic and metabolomic analysis to understand the molecular responses to water deficit in a marker-class common bean accession cultivated under N_2-fixation or fertilized with nitrate (NO_3^-). RNA-seq revealed more transcriptional changes in the plants fertilized with NO_3^- than in the N_2-fixing plants. However, changes in N_2-fixing plants were more associated with drought tolerance than in the NO_3^- fertilized ones. N_2-fixing plants accumulated more ureides in response to drought and GC/MS and LC/MS analysis of primary and secondary metabolites profiles revealed that N_2-fixing plants also had higher levels of ABA, proline, raffinose, amino acids, sphingolipids and triacylglycerols than the NO_3^- fertilized ones. Moreover, plants grown under nitrogen fixation recovered from drought better than plants fertilized with NO_3^-. Altogether we show that common bean plants grown under symbiotic nitrogen fixation were more protected against drought than the plants fertilized with nitrate.

CHAPTER 4

CRISPR/CAS9 EDITING OF TWO COMMON BEAN ADENINE PHOSPHORIBOSYL TRANSFERASE GENES REVEALS FUNCTIONAL SPECIALIZATION OF ADENINE SALVAGE PROTEINS

Chapter 4. CRISPR/Cas9 editing of two common bean adenine phosphoribosyl transferase genes reveals functional specialization of adenine salvage proteins.

Abstract

Gene editing using CRISPR/Cas has become a useful tool for gene functional analysis and crop improvement. Regulation of the purine nucleotides pool is particularly important for common bean (*Phaseolus vulgaris*) crop productivity since this legume uses ureides derived from purine nucleotides as the primary nitrogen storage and export molecules. Purine nucleotides can be synthesized de novo or recovered through the salvage pathways. Adenine phosphoribosyl transferase (APRT) is the enzyme dedicated to the salvage of the adenine nucleobase for purine nucleotide synthesis and is known to also act on the conversion of active to inactive cytokinins. Expression levels, biochemical characterization, and subcellular location of the four APRT isoforms from common bean suggest functional differences. CRISPR/Cas9 targeted downregulation of the two main APRT isoforms, followed by the metabolomic and physiological analysis of the transgenic hairy roots reveal that although the two proteins have redundant functions, PvARPT1 mainly participates in the salvage of adenine, whereas PvAPRT5 is the predominant form in the regulation of cytokinin homeostasis, root and nodules growth and stress responses.

Introduction

Common bean (*Phaseolus vulgaris*) is one of the most important legumes for human consumption due to its nutritional value, with high content in proteins, vitamins and minerals (Mukankusi *et al.*, 2019). Common bean also has a great environmental value, since legumes fix atmospheric nitrogen (N_2), which reduces the use of fertilizers allowing a more economical and sustainable agriculture. These advantages increase the need improve the yields of this crop that is highly constrained by stress. Common bean belongs to the group of ureidic legumes, which use the nitrogen fixed in nodules for the *de novo* synthesis of purine nucleotides. Then, purine nucleotides are oxidized to produce ureides (allantoin and allantoate), which are the main transport and storage molecules of nitrogen in these plants (Atkins, 1991; Díaz-Leal *et al.*, 2012; Werner and Witte, 2011). Purine nucleotides are essential for the productivity of this crop. Not only

because purine nucleotides are components nucleic acids as well as in energy metabolism molecules, but also, they are the precursors of ureides (Zrenner *et al.*, 2006; Zrenner and Ashihara, 2011). Thus, knowledge concerning the regulation of purine nucleotide levels could help to develop strategies to obtain common beans that are better adapted to adverse conditions.

Levels of purine nucleotides are strictly controlled through the regulation of its *de novo* synthesis, recycling, and degradation pathways. Purine nucleotides are primarily synthesized *de novo* in most organisms (Berens *et al.*, 1995). This pathway involves ten enzymatic reactions that begin with the formation of phosphoribosylamine from phosphoribosyl pyrophosphate (PRPP) and glutamine, ending with the formation of Adenosine-5′-monophosphate (AMP) and guanosine-5′-monophosphate (GMP) (Coleto *et al.*, 2016; Zrenner *et al.*, 2006). However, *de novo* synthesis is less energetically favourable than salvage pathways of purine nucleotides. Salvage of purine bases and nucleosides to purine nucleotides is not only complementary to *de novo* synthesis, but it has been proven essential for plant growth and metabolism (Ashihara *et al.*, 2018; Moffatt and Ashihara, 2002; Witte and Herde, 2020).

Salvage of purine nucleobases to nucleotides depends on the activity of two enzymes, hypoxanthine/guanine phosphoribosiltransferse (HGPRT; 2.4.2.8) and adenine phosphoribosyltransferase (APRT; EC 2.4.2.7). Deficiency in APRT in humans lead to accumulation of 2,8-dihydroxyadenine (DHA), generating kidney stones and in some cases crystalline nephropathy (Bollée *et al.*, 2012), while deficiency in HGPRT causes the Lesch-Nyhan syndrome, associated with uric acid overproduction and a continuum spectrum of neurological manifestations depending on the degree of the deficiency (Torres and Puig, 2007). In addition, HGPRT and APRT proteins are of great importance for ATP production, necessary for the proper functioning of neural connections in animals (Frenguelli, 2019). In plants the adenine nucleobase is present at much higher levels than the guanine nucleobase (Ashihara *et al.*, 2018; Witte and Herde, 2020). Thus, although the enzymatic activity of HGPRT is highly relevant in mammalian tissues, in plants, APRT activity levels are higher than those of HGPRT and APRT seems to be of higher physiological importance (Adams and Harkness, 1976; Deng and Ashihara, 2010).

The APRT enzyme was first isolated from yeast by (Kornberg *et al.*, 1955) and, later from other organisms including plants (Moffatt *et al.*, 1994). This enzyme catalyses the reaction between adenine and phosphoribosyl pyrophosphate (PRPP) to form AMP

(Figure 1a) and, although APRT activity has been associated with a single protein isoform in mammals (Tischfield and Ruddle, 1974), five APRT isoforms have been described in *Arabidopsis thaliana* (Allen *et al.*, 2002). Indeed, the same enzyme has been shown to act in the activation and inactivation of cytokinin bases, trans-zeatin, isopentenyladenine and benzyl adenine (Allen *et al.*, 2002; Moffatt and Ashihara, 2002; Mok and Mok, 2001; Zhang *et al.*, 2013). Therefore, APRT activity is not only important in nucleotides and energy metabolism, but also in regulation of plant growth and development.

The current research shows the molecular and functional characterization of APRT enzyme in the ureidic legume common bean. For this purpose, a bioinformatic analysis of the amino acid and peptide sequences of the genes encoding APRT protein in common bean has been carried out. Differences in biochemical properties, subcellular location, and gene expression and APRT activity levels in different common bean suggest functional specialization of the different isoforms. Mutated hairy roots of the two genes with the highest expression in roots and nodules (*PvAPRT1* and *PvAPRT5*) have been obtained by gene editing using CRISPR/Cas9 technology. The physiological and metabolomic analysis of these transgenic roots revealed that although the PvARPT1 mainly participates in the purine nucleotide salvage pathway, the PvAPRT5 could function in the regulation of cytokinin levels.

Material and methods

Plant material and culture conditions

Phaseolus vulgaris of the marker class Great Northern "Matterhorn" (PMB-0220) was used in this study. *P. vulgaris* seeds were sterilized in ethanol (100%) for 30 s, incubated in sodium hypochlorite (5%) for 5 min and washed five to six times with sterile water. Then, seeds were placed on wet sterile paper in Petri dishes for 72 h. Germinated seeds were sown in pots with a mix of vermiculite and perlite w/w (2/1), with three plants per pot. Plants were inoculated twice, at sowing and 7 days after sowing, with a fresh culture of *Rhizobium leguminosarum* ISP14. A group of plants was watered every third day with nutrient solution (Rigaud and Puppo, 1975) without nitrogen and other group of plants was watered with the same solutions but containing 10 mM NO_3^-. Plants were cultured under standard growth chamber conditions, with 300 $\mu E.m^{-2}.s^{-1}$ lighting for 16 h at 26˚C and 8 h of darkness at 20˚C and 70% relative humidity for 28 days. Plant material was

collected, frozen and pulverized with liquid nitrogen and stored at -80°C until its use for gene expression and biochemical analysis.

Identification, cloning and sequence analysis of common bean APRTs

Genes coding for the common bean APRT protein were identified using sequences of the *APRT* genes from *Arabidopsis thaliana*. A BLAST analysis was performed between APRT peptide sequences from *A. thaliana* and translated genomes of *P. vulgaris* v2.1 on Phytozome v13 (https://phytozome-next.jgi.doe.gov/) (Goodstein *et al.*, 2012; Schmutz *et al.*, 2014). Sequences coding APRTs from common bean were used to design gene specific primers to amplify each of the four putative PvAPRTs from PMB-220 common bean genotype (Supplementary Table S1).

Full-length *Phvul.009G070900* (*PvAPRT1*), *Phvul.001G050500* (*PvAPRT2*), *Phvul.002G061600* (*PvAPRT4*) and *Phvul.009G092200* (*PvAPRT5*) coding sequences were amplified using specific primers containing cleavage sites for *Cla I* and *Sma I* restriction enzymes (Supplementary Table S1). For amplification, VELOCITY DNA Polymerase (BIOLINE) was used, following manufacturer´s instructions. DNA fragments were cloned in pSpark II vector following the pSpark® II DNA cloning kit (Canvax) manufacturer´s instructions. *E. coli* (*DH5α*) competent cells were transformed with the product resulting from each ligation and positive colonies were selected after PCR screening and plasmids were isolated. Cloned inserts of full-length *PvAPRT* were confirmed by sequencing.

Phylogenetic analysis, physicochemical properties, and subcellular location prediction

The peptide sequences of APRT protein isoforms were obtained by translation using the verified sequences from *P. vulgaris* Great Northern "Matterhorn" (PMB-0220) and compared with APRTs available sequences from *Glycine max*, *Medicago truncatula*, *Lotus japonicus*, *Oryza sativa*, *Arabidopsis thaliana*, *Chlamydomonas reinhardtii*, *Homo sapiens*, as well as other available sequences from various *P. vulgaris* genotypes. Sequences from plants were retrieved from Phytozome 13 website (https://phytozome-next.jgi.doe.gov/) (Goodstein *et al.*, 2012; Schmutz *et al.*, 2014). The alignment of the peptide sequences of APRT protein was done using the DNASTAR Lasergene and CLUSTAL software (https://www.ebi.ac.uk/Tools/msa/clustalo/), and the phylogenetic analysis was made using the Neighbour joining method (MEGA 11).

Physicochemical properties such as isoelectric point, molecular weight (kDa), stability

and aliphatic index of deduced APRT proteins from PMB-0220 common bean line were calculated using ProtParam Tool (https://web.expasy.org/protparam/). The possible subcellular location of each APRT protein was searched using the TargetP software (http://www.cbs.dtu.dk/services/TargetP/).

Analysis of Cis- regulatory motives in PvAPRT promoter sequences

The proximal upstream sequences (1500 pb) of the transcription start of each PvAPRT gene from *Phaseolus vulgaris* UI111 v1.1 were obtained from Phytozome v13 (https://phytozome-next.jgi.doe.gov/). The *in-silico* analysis of the promoter sequences was done using PlanCare prediction software (Lescot *et al.*, 2002).

Expression of recombinant PvAPRT-HA-Strep or -YFP tagged proteins in Nicotiana benthamiana

Coding sequence of the full-length cDNAs coding PvAPRT1, PvAPRT2, PvAPRT4 and PvAPRT5 were excised from the SparkII-APRT vectors using the *Cla I* and *Sma I* sites and cloned into pXCSHAStrepII to generate C-terminal hemagglutinin (HA)- and Strep-tagged proteins (Witte *et al.*, 2004) or pXCS-YFP for C-terminal yellow fluorescent protein (YFP)-tagged proteins (Chen and Witte, 2020). Constructs were verified by sequencing and cells of *Agrobacterium tumefaciens* (GV3101::pMP90RK) were transformed by electroporation with each of the four pXCS:PvAPRT-HAStrep or pXCS:PvAPRT-YFP plasmids.

A. tumefaciens cells with each pXCSHAStrep:PvAPRT and pXCHAStrep-YFP:PvAPRT expression constructs, and *A. tumefaciens* (C58C1::pCH32), with Pbin61::P19 expressing the P19 silencing suppressor, were cultured overnight at 28°C, collected by centrifugation at 1100 *g* for 10 min and resuspended in induction medium (10 mM $MgCl_2$; 10 mM MES, pH 5.6 and 150 µM acetosyringone). After an incubation at room temperature for 2 h, cells suspensions were diluted with induction medium until 0.5 OD_{600nm} and mixed 1:1 with the Pbin61:P19 similarly diluted cells. The mix of PvAPRTs and P19 construct containing cells was used to infiltrate leaves from 3-week-old *N. benthamiana*. At 4 days after agroinfiltration, tissue was collected and stored at -80°C for further analysis or used directly for fluorescence and protein localization by confocal microscopy, using an Axioskop 2 MOT microscope (Carl Zeiss, Jena GmbH, Germany) equipped with a Krypton and an Argon laser, controlled by Carl Zeiss Laser

scanning System LSM5 PASCAL software (Carl Zeiss).

Gel electrophoresis and western blot analysis

Proteins were separated by 12% SDS-PAGE (Laemmli, 1970) at 100 V, using a Mini PROTEAN III system (Bio-Rad). Proteins were electro-transferred to polyvinylidene fluoride (PVDF) membrane (Sigma) at 0.3 A for 3 h. After electroblotting, PVDF membranes were incubated overnight with monoclonal antibodies against -GFP (Sigma) or HA (Thermo Fisher Scientific) at a 1:2500 dilution and 4˚C. Anti-mouse IG conjugated with alkaline phosphatase (Sigma) as used as secondary antibody at a 1:12000 dilution. Reaction for immunodetection was developed by phosphatase activity (0.1 mM Tris-HCl (pH 9.5); 0.15 $mg.ml^{-1}$ BCiP; 5 mM $MgCl_2$ and 0.30 $mg.ml^{-1}$ NBT).

RNA isolation and gene expression analysis.

RNA extraction was done using 50-100 mg of frozen plant tissue. One ml of Trizol (Nzyol Tzytech) was added to the pulverized frozen tissue, homogenised, and incubated for 5 min at room temperature. Then, 200 µl of chloroform/isoamyl (24/1) were added, homogenized by vortex, incubated 8 min at room temperature and centrifuged for 10 min at 11000 g at 4˚C. About 500 µl of aqueous phase were collected and mixed with 0.8 vol of isopropanol in a new tube. Samples were incubated 5 min at room temperature and centrifuged at 11000 *g* for 15 min and at 4˚C. Aqueous phase was removed, and 1 ml of ethanol (75%) was added to the RNA pellet and centrifuged 5 min at 7500 *g* at 4˚C. The supernatant was carefully removed, and the RNA was resuspended in 200 µl of miliQ water. Next, 132 µl of lithium chloride 8 M were added and samples were incubated overnight at 0˚C for a second precipitation of the RNA. After incubation, RNA samples were centrifuged at 11000 g for 20 min at 4˚C and the supernatant was removed. One ml of ethanol (75%) was added, and samples were centrifuged at 7500 *g* for 5 min at 4˚C and the supernatant was removed. RNA pellets were let dry for several minutes and resuspended in 40 µl of miliQ water. Quantity and purity were measured using a NanoDrop spectrophotometer and by observation after agarose gel electrophoresis.
Genomic DNA was removed from RNA samples using DNase I (New England Biolabs) at 37˚C for 10 min. Synthesis of cDNA was done using 2.5 µg of DNase-treated RNA and iScript™ (Bio-Rad) reverse transcriptase, following the manufacturer´s instructions. Gene expression analysis was made by qRT-PCR using iQ SYBR-Green supermix (Bio-Rad) using specific primers for each gene (Supplementary Table S1) in an iCycler iQ

System (Bio-Rad). The relative expression of each gene encoding APRT protein in *P. vulgaris* tissues was normalized to that of *Actin-2* or *Ubiquitin* constitutive genes and estimated according to (Livak and Schmittgen, 2001).

Purification of PvAPRT-HA-Strep protein

Recombinant PvAPRT-HA-Strep proteins were purified by affinity binding to StrepTactin Sepharose (GE Healthcare) according to (Witte *et al.*, 2004). Briefly, 0.40 g frozen and crushed leaf tissue of *N. benthamiana* was homogenized with 800 µl of extraction buffer (100 mM Tea-NaOH (pH 8); 150 mM NaCl; 5mM DTT and 1 mM 0.5 % Triton-X-100), centrifuged for 10 min at 15000 *g* and 4˚C and 40 µl StrepTactin Sepharose were added to the supernatant and incubated with mild shaking for 10 min at 4˚C. The suspension was centrifuged for 30 s at 700 *g*. After centrifugation, the supernatant was removed, and the pellet was washed 5 times with 500 µl of wash buffer (100 mM Tea-NaOH (pH 8); 150 mM NaCl; 5 mM DTT and 1 mM 0.5 % Triton-X-100). Purified protein was eluted with 100 µl of elution buffer (100 mM Tea-NaOH (pH 8); 150 mM NaCl; 5 mM DTT; 1 mM Triton-X-100 (0.5 %) and 2.5 mM d-Desthiobiotin (Sigma) was added, and this mix was incubated 5 min at room temperature with slight agitation. After incubation, it was centrifuged at 700 *g* for 1 min and supernatant containing the purified protein was collected.

APRT activity assay

Adenine conversion to AMP of recombinant purified PvAPRT proteins and crude extracts from several plant tissues was determined by the method described by (Kojima *et al.*, 1991) with minor modifications to adapt it to plant tissues. Crude extracts from tissue samples were prepared with 60 mg of tissue, mixed and homogenized with extraction buffer (100 mM Tea-NaOH (pH 8); 5 mM DTT and 1mM $MgCl_2$) in a relation 1:3. Extracts were centrifuged at 4˚C for 10 min and the supernatant was dialyzed through a SpinTrap G25 column, previously equilibrated with extraction buffer and centrifuged for 30 s at 800 *g*. APRT activity assay was carried out by two stepwise coupled enzymatic reactions. The first reaction catalyzed by APRT transforms adenine and phosphoribosyl pyrophosphate (PRPP) to AMP and pyrophosphate. For it, 50 µl of dialysed sample was mixed with 500 µl of reaction buffer I (50 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 1 mM PRPP and 1 mM Adenine) and incubated for 1 hour at 37˚C. The reaction was stopped by boiling at 100˚C for 5 min and centrifuged for 10 min at 3000

g at 4˚C, and the supernatant was collected.

In the second reaction, AMP generated in the first reaction was transformed to inosine. 300 µl of the first reaction supernatant was mixed with 500 µl of reaction buffer II (100 mM Tris-HCl, pH 9; 10 mM $MgCl_2$) and 1 $U.ml^{-1}$ of Calf Intestinal Alkaline Phosphatase (CIAP) (Canvax) and 1.5 $U.ml^{-1}$ adenosine deaminase (ADA) (Sigma-Roche). The mix was incubated 1 hour at 37˚C. After incubation, proteins were precipitated with 50 µl of 3.6 M $HClO_4$ and centrifuged at 3000 *g* at 4˚C for 10 min. The resulting inosine was measured by HPLC (Jasco) equipped with AS-2055 Plus autosampler, PU 2089 pump, Jasco MD-2010 Plus Diode Array, and a 250 mm x 4.6 mm Extrasil ODS2 column (C18. 5 µm). Samples (20 µl) were resolved using an isocratic eluent with 50 mM sodium phosphate buffer pH 2.3 and 2 % methanol for 55 min at a flow rate of 0.5 $ml.min^{-1}$.

The assay using zeatin and phosphoribosyl pyrophosphate (PRPP) to transform zeatin nucleobase to zeatin nucleotide and pyrophosphate by APRT activity was done using 1-3 µl of pure APRT1 and APRT5, 500 µl of reaction buffer I (50 mM Tris-HCl, pH7.5; 5 mM $MgCl_2$; 1 mM PRPP and 50 µM zeatin) and incubated for 1 hour at 37˚C. The reaction was stopped at 100˚C for 5 min and centrifuged 10 min at 3000 *g* at 4˚C, and the supernatant was collected and lyophilized. Lyophilized samples were used for hormone metabolite analysis as explained below.

Generation of CRISPR/Cas9 targeted mutants of PvAPRT1 and PvAPRT5 genes

Gene editing with CRISPR/Cas9 to mutate *PvAPRT1* and *PvAPRT5* genomic sequences was done by using Breaking-Cas software to select the target gene edition sites (https://bioinfogp.cnb.csic.es/tools/breakingcas/) (Oliveros *et al.*, 2016). Sequences with no or low off-target predictions were selected to the design of each single guide RNA (sgRNA), and the pUC119-gRNA and pFGC-pcoCas9 vectors (Addgene) were used to synthesize the guide RNAs and to generate the CRISPR/Cas9 gene editing constructs, respectively (Li *et al.*, 2013). Two sgRNAs were generated to target first and third exon in PvAPRT1 and second and third exon positions in PvAPRT5 genomic sequences.

The first guide was made with two PCR rounds, using pUC119-gRNA as a template, specific primers for the target sequences and Phusion Hot Start II High-Fidelity PCR Master Mix (Thermo Scientific). The primers used for the first PCR round were F1-U6-AscI and R1-APRT1-1 for PvAPRT1-Cas9 and F1-U6-AscI and R1-APRT5-712 for

PvAPRT5-Cas9. The second PCR was made with F2-APRT1-1 and gRNA-PacI-R primers for PvAPRT1-Cas9 and F2-APRT5-712 and gRNA-PacI-R for PvAPRT5-Cas9 (Supplementary Table S1). The two PCR amplicons were purified and used as templates for a recombinant PCR, using the primer pairs F1-U6-AscI and gRNA-PacI-R, whose product was purified and inserted in pFGC-pcoCas9 vector using *AscI* and *PacI* as restriction enzymes. The pFGC-pcoCas9 vector with the first guide was used to transform *E. coli* DH5α cells and plasmid was isolated from positive colonies.

The second guide was made using the same approach as for the first guide PCR. The pair of primers used were F1-U6-SwaI and R1-APRT1-2 for PvAPRT1-Cas9 and F1-U6-SwaI and R1-APRT5-1527 for PvAPRT5-Cas9 for the first PCR round. The second PCR was made with F2-APRT1-2 and gRNA-AscI-R primers for PvAPRT1-Cas9 and F2-APRT5-1527 and gRNA-SwaI-R primers for PvAPRT5-Cas9. Then, the two PCR products were used as template for the recombinant PCR using F1-U6-SwaI as forward primer and gRNA-AscI-R gRNA-SwaI-R as reverse primers for APRT1 and 5 respectively. The final PCR products were purified and inserted in pFGC-pcoCas9 vector containing the previously inserted first guide, using *AscI* and *SwaI* as restriction enzymes for PvAPRT1-Cas9, and *SwaI* as restriction enzyme for PvAPRT5-Cas9. Afterward, pFGC-pcoCas9 vector with both guides was transformed in Stellar™ Competent Cells (TaKaRa). The resulting constructs were verified by sequencing using specific primers for the APRT sequences (Supplementary Table S1) and introduced into the strain K599 of *Rhizobium rhizogenes* by electroporation.

RNAi Silencing of PvAPRT1 and PvAPRT5.

To generate the silencing hairpin RNA construction, fragments from the 3´-region of *PvAPRT1* and *PvAPRT5* cDNAs were cloned into pFGC5941 vector (AY310901) (Kerschen *et al.*, 2004). For that, 353 base pairs *PvAPRT1* transcript coding DNA and 331 base pairs of that from *PvAPRT5*, were amplified by PCR using primers containing *SwaI* and *AscI* restriction sites for the direct fragment, and *XbaI* and *BamHI* for the reverse sequence for each gene fragment (Supplementary Table S1). The resulting PCR products were cloned into pFGC5941 vector using *SwaI* and *AscI* sites in sense orientation upstream of an intron, and the resulting PCR products with *XbaI* and *BamHI* sites were inserted in antisense orientation, downstream the intron. The resulting constructs were verified by sequencing and used to transform *R. rhizogenes strain* K599 by electroporation.

Generation of common bean transformed hairy roots

Hairy roots transformation with the CRISPR/Cas9-APRTs or with the RNAi-APRTs constructs was made following the protocol described by (Estrada-Navarrete *et al.*, 2007). Common bean stems were slightly punched below the cotyledonary nodes and *R. rhizogenes* suspension was infiltrated in the wounds. The inoculated zone was maintained under high humidity for several days, spraying irrigation solution with kanamycin until hairy roots start to emerge from the damaged stems. At 10 days after agroinfiltration, the stem was cut to remove all original roots, and the plants having only the hairy roots that appeared from the infection with *R. rhizogenes* were shown on perlite and vermiculite (1:2 v/v) substrate. Plants were inoculated at sowing and reinoculated one week later with *R. leguminosarum* (CIAT-899) for nodulation, and grown under nitrogen fixation conditions, irrigated with the nutritive solution without nitrogen. After 28 days of transplantation, the corresponding plant material was collected and analysed. Individual hairy roots were collected, although some small roots were also pooled. Expression levels of the APRTs was analysed by qRT-PCR and roots with reduced expression of the two targeted genes were chosen for further analysis.

Metabolite identification and quantification

Metabolomic analysis of transgenic root samples was performed following the protocol described by (Salem *et al.*, 2020). Briefly, 50 mg of previously pulverized freeze-dried tissue from 6 independent hairy roots per condition was used. Metabolites were extracted with 1 ml of methyl-tert-butyl-ester:methanol (3:1). Hydrophobic and polar fractions were aliquoted by previous fractionation through phase separation adding H_2O:MeOH (3:1 v/v). Specifically, from the hydrophobic phase, 230 µl were obtained for hormones analysis, whereas 130 µl and 260 µl from the polar fraction were taken for primary and secondary metabolism, respectively. Then, each fraction was concentrated in a speed vac and pellets were kept at -80˚C until use.

Gas chromatography coupled with mass spectrometry (GC/MS)

To analyse primary metabolism compounds, pellets from 130 µl of polar fraction were derivatized by mixing with 40 µl of 20 $mg.ml^{-1}$ methoxyamine hydrochloride in pyridine and incubated 120 min at 37˚C. Subsequently, 70 µl N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were added and samples were incubated

under shaking for 30 min at 37˚C (Lisec *et al.*, 2006). An autosampler Gerstel Multi-Purpose system (Gerstel GmbH & Co.KG, Mülheim an der Ruhr, Germany) was used to inject the samples into a gas chromatograph coupled to a time-of-flight mass spectrometer (GC-MS) system Leco Pegasus HT TOF-MS (LECO Corporation, St. Joseph, MI, USA). Helium was used as carrier gas at a constant flow rate of 2 $ml.s^{-1}$ and gas chromatography were performed on a 30 m DB-35 column. The injection temperature was 230˚C and the transfer line and ion source were set to 250˚C. The initial temperature of the oven (85˚C) increased at a rate of 15˚C/min up to a final temperature of 360˚C. After a solvent delay of 180 s mass spectra were recorded at 20 scans s^{-1} with m/z 70-600 scanning range. Mass chromatograms were evaluated using Chroma TOF 4.5 (Leco) and TagFinder 4.2 software. Metabolite identification and annotation defined in Supplementary Table S2 were performed as reporting standards (Alseekh *et al.*, 2021).

Liquid chromatography coupled with mass spectrometry (LC/MS)

To analyse the secondary metabolites, 260 µl desiccated polar fraction were resuspended in 400 µl of 50% MeOH by vortex for 5 min. Samples were sonicated for 3 min and centrifuged for 5 min. Then, samples were run on a UPLC-LC-MS machine equipped with an HSS T3 C18 reverse-phase column (100 × 2.1 mm internal diameter, 1.8 µm particle size; Waters) that was operated at a temperature of 40˚C. The mobile phases consisted of 0.1% solvent A (formic acid in water) and 0.1% solvent B (formic acid in acetonitrile). The flow rate of the mobile phase was 400 $\mu l.min^{-1}$, and 2 µl of sample was loaded per injection. The UPLC instrument was connected to an Exactive Orbitrap-focus (Thermo Fisher Scientific) via a heated electrospray source (Thermo Fisher Scientific). The spectra were recorded using full-scan positive and negative ion-detection mode, covering a mass range from m/z 100 to 1,500. The resolution was set to 70,000, and the maximum scan time was set to 250 ms. The sheath gas was set to a value of 60 while the auxiliary gas was set to 35. The transfer capillary temperature was set to 150˚C while the heater temperature was adjusted to 300˚C. The spray voltage was fixed at 3 kV, with a capillary voltage and a skimmer voltage of 25 V and 15 V, respectively. MS spectra were recorded from minutes 0 to 19 of the UPLC gradient. Processing of chromatograms, peak detection, and integration were performed using RefinerMS (version 5.3; GeneData). Metabolite annotation in Supplementary Table S2 were performed as in (Alseekh *et al.*, 2021). When using the in-house reference compound

library we allowed for 10 ppm mass error, and a dynamic retention-time shift of 0.1.

Metabolomic data processing and statistical analysis

Up to six independent samples were used in the metabolomic analysis for each condition: Control, transgenic root with empty pFGC-pcoCas9; CRISPR/Cas9 APRT1 and CRISPR/Cas9 APRT5, transgenic roots with the *PvAPRT1* and *PvAPRT5* genes mutated by CRISPR/Cas9 technology. Metabolomic analysis was made using the website MetabolAnalist 5.0 (https://www.metaboanalyst.ca/) (Pang *et al.*, 2021) and Expressionist Analyst 14.0.5 (Genedata, Basel, Switzerland) (https://www.genedata.com/products/expressionist).

Experimental design and statistical analysis of the data

Analysis of expression levels and APRT activity in common bean tissues was done using 3 independent replicates from a pool of 3 plants per replicate (a total of 9 plants analyzed for each condition) grown under the conditions described in the section on vegetal material. Student's *t-test* was performed using the Holm-Sidak method.

For the *PvAPRT1* and *PvAPRT5* targeted by CRISPR/Cas9, a total of 16 plants with transgenic roots at each condition were tested. From each plant, the expression levels of individual hairy roots were analysed to select the silenced roots ($n \geq 30$). Next, APRT activity levels and metabolomic analysis ($n \geq 6$) were determined using transgenic roots pools samples previously selected as APRT downregulated.

Statistical analysis was performed using ANOVA with the Tukey´s tests, using GraphPad Prism 8 software package to calculate the statistical significance and fold change of the metabolites for each transformed tissue.

Results

Peptide sequence and phylogenetic relationships analysis

Four APRT coding genes were identified in the *P. vulgaris* v2.1 genome: *Phvul.009G070900* (*PvAPRT1*), *Phvul.001G050500* (*PvAPRT2*), *Phvul.002G061600* (*PvAPRT4*) and *Phvul.009G092200* (*PvAPRT5*) using the APRT sequences from *Arabidopsis* as a bait and named according to their respective homology to the five *Arabidopsis* APRTs. Using this information, the four full-length *PvAPRT* coding sequences from the *P. vulgaris* Matterhorn (PMB-0220) genotype were cloned and the

peptide sequences were analysed. The four APRT proteins in *P. vulgaris* (PMB-0220) shared a high degree of homology and contained the phosphoribosyl transferase (PRT)-type I conserved domain. However, phylogenetic analysis classified them into two homology groups (Supplementary Figure S1a and S2a). PvAPRT1 protein showed greater homology with PvAPRT4, both with a possible chloroplast transfer signal peptide, than with the other two proteins, whereas the two proteins in the second group, PvAPRT2 and PvAPRT5, did not shown any transfer signal peptide, and APRT5 contain a valine instead of the conserved lysine residue in the last position of the conserved active site (Figure 1c and Supplementary Figure S1b and S2b). However, comparison of the APRT sequences of three common bean genotypes revealed that PvAPRT1 and 4 proteins are grouped in the same cluster even if they lack the signal peptide, as it happens in APRT4 homologue (*PvUI111.02G061000*) from *Phaseolus vulgaris* UI111 v1.1 genotype (Supplementary Figure S2).

Phylogenetic comparison of the APRTs proteins sequences in several organisms revealed that, soybean, an ureidic legume as common bean, has 13 genes encoding APRTs isoforms. In contrast, four APRT coding sequences were found in *M. truncatula*, an amidic legume. Of these four genes, three appeared close to those encoding the APRT1, APRT4 and APRT5 isoforms of *P. vulgaris*, while one of them appeared as phylogenetically closer to the gene encoding the APRT3 isoform in *A. thaliana* (Figure 1b). As in *A. thaliana*, five genes coding for the APRT protein were found in rice, whereas only two copies of APRT encoding genes were found in the green algae *Chlamydomonas*, and a single gene, although with two transcript variants, encode the APRT isoforms in humans. Surprisingly, three of the 13 soybean APRTs sequences appeared closer to the human and algae proteins than to any other plant isoforms (Figure 1b).

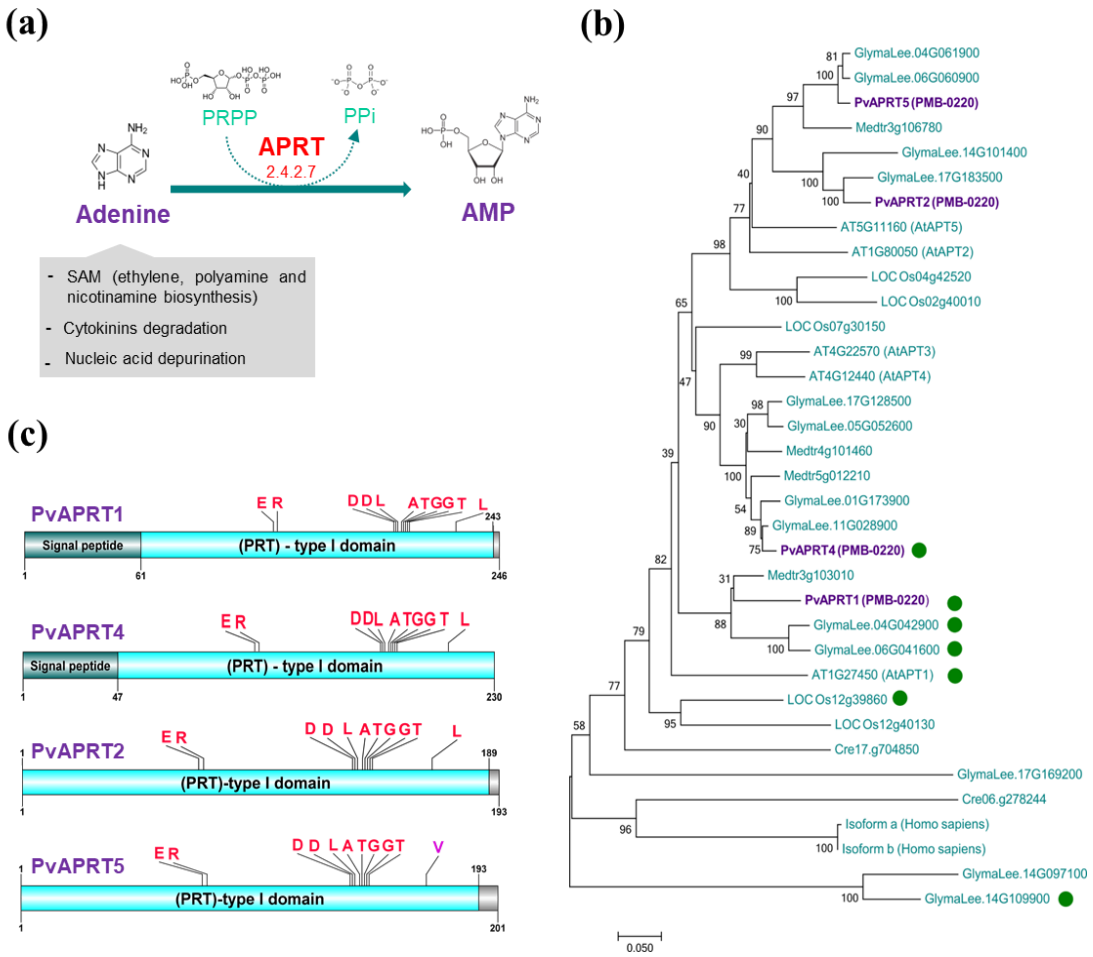

Figure 1. Phylogenetic distances of APRTs proteins. (a) Schematic representation of the enzymatic reaction catalyzed by the APRT protein. (b) Evolutionary relationships of APRTs. Sequences were aligned using the Clustal W method and evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with sum of branch length = 4.24 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. The analysis involved 35 amino acid sequences belonging to *Homo sapiens* (isoform a and b), *Phaseolus vulgaris*, *Glycine max*, *Medicago truncatula*, *Oryza sativa*, *Arabidopsis thaliana and Chlamydomonas reinhardtii*. All ambiguous positions were removed for each sequence pair. There was a total of 771 positions in the final dataset. Evolutionary analyses were conducted in MEGA 11. Green circle shows the APRT isoforms with signal peptide to the chloroplast. (c) Organization of peptide domains in the APRT proteins from *P. vulgaris*, where red and purple letter denote invariable residues at the active site.

Subcellular location and biochemical characterization of PvAPRTs proteins expressed in Nicotiana benthamiana

The full-length PvAPRT1, PvAPRT2, PvAPRT4 and PvAPRT5 peptide sequences were 246, 193, 230 and 201 amino acids long, with an estimated molecular mass of 26.93, 20.9, 25.37 and 21.84 kDa, respectively (Supplementary Table S3). Removal of APRT1 and APRT4 signal peptides according to Psort prediction led to mature proteins of 186 and 184 amino acids with molecular weights of 20.44 and 20.36 kDa, respectively. Thus, the *in-silico* analysis suggested that the four mature APRT isoforms have similar molecular weights, although two of them seem to perform their function in the chloroplast while the other two appear to do so in the cytosol. To determine the cellular location of the four proteins from *P. vulgaris*, APRT-YFP-fusions were overexpressed in *N. benthamina* leaves. Western blots, performed with crude extracts from agroinfiltrated *N. benthamiana* leaves, showed the presence of bands of approximately 50 kDa, which confirmed that each isoform of the APRT protein (~20 kDa) was bound to the fluorescent protein YFP (~27 kDa) (Supplementary Figure S3). *N. benthamiana* leaves agroinfiltrated with each construction observed under a confocal microscope confirmed that APRT1 and APRT4 localized in chloroplasts, although a small amount of APRT4 protein was also observed in the cytosol (Figure 2a). On the other hand, the APRT2 and APRT5 isoforms, lacking any targeting signal peptides in their amino acid sequences, were found in the cytoplasm (Figure 2a).

Construct of C-terminal HA-Strep-tagged PvAPRT1, PvAPRT2, PvAPRT4 and PvAPRT5 were expressed in *N. benthamiana* leaves. Western blot against the HA epitope in crude leaf extract and after affinity purification confirmed the expression of proteins with ~23 kDa apparent molecular weight, corresponding to the expected size of the recombinant PvAPRT isoforms, from which PvAPRT4 appeared as slightly smaller than the other APRTs, despite the similar amino acid length of the four mature proteins (Figure 2b, c and Supplementary Figure 3c, e).

On the other hand, an HPLC enzymatic assay for APRT activity was optimized for plant tissues using the extracts from *N. benthamiana* tissues expressing the APRT-Ha-Strep isoforms. Activity using adenine and PRPP as enzyme substrates was higher in the *N. benthamiana* extracts overexpressing PvAPRT1, than those with PvAPRT4 and PvAPRT5 proteins, whereas activity was absent in extracts from PvAPRT2 expressing tissues (Figure 2d). Same pattern of activity levels was found when purified

recombinant APRTs were used in the enzymatic assay. Michaelis-Menten constants were determined for the purified PvAPRT1, PvAPRT4 and PvAPRT5. The Km constants for adenine were 0.359, 0.0071 and 0.0062 mM, whereas the Km for PRPP were 0.92; 0.23 and 0.097 mM for PvAPRT1, PvAPRT4 and PvAPRT5, respectively

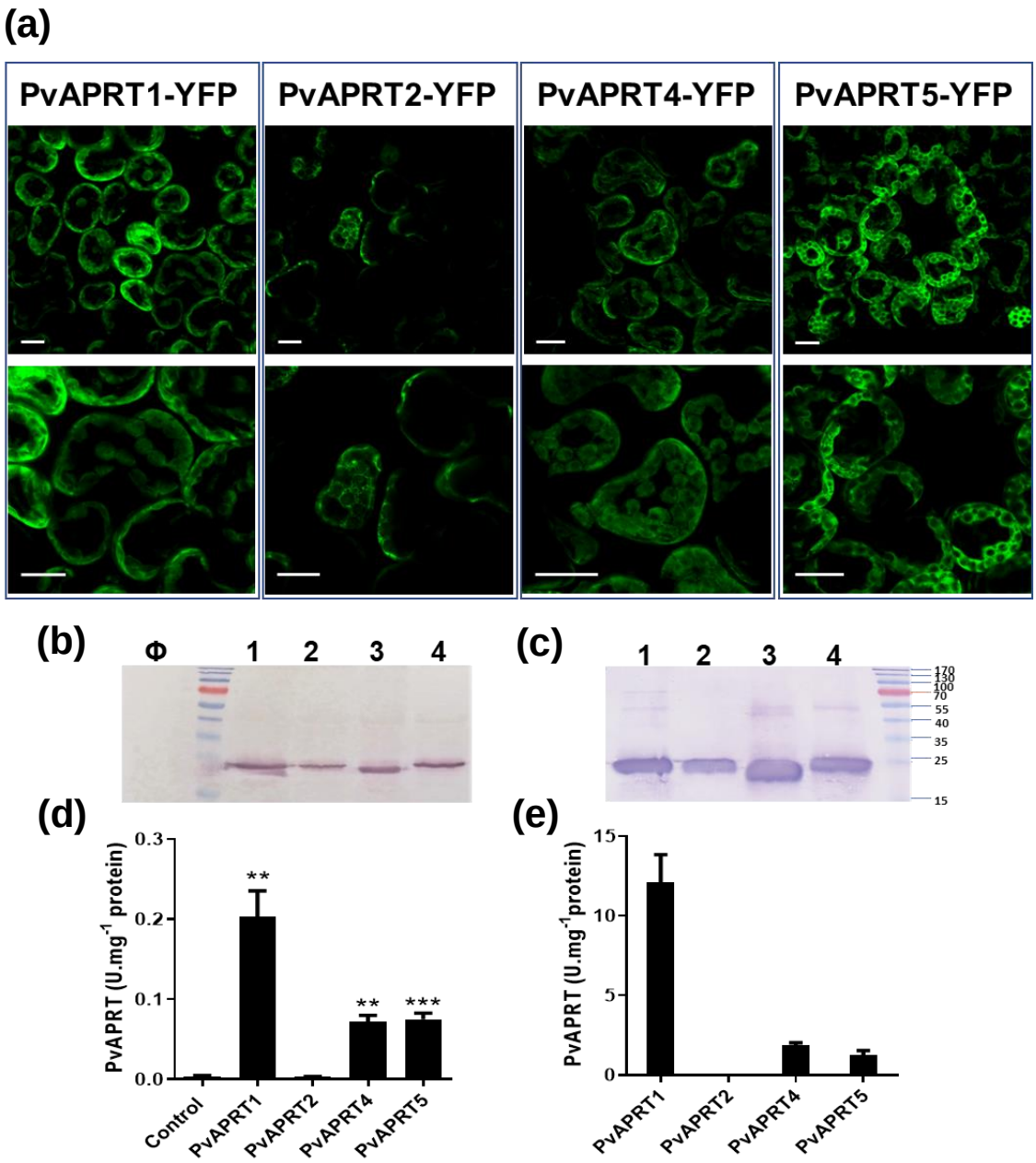

Figure 2. Characterization of recombinant PvAPRT isoforms expressed in *N. benthamina* leaves. (a) Confocal microscopy images of *N. benthamiana* leaves expressing the four PvAPRTs labeled with YFP. The white bar indicates 20 µM. (b) Western-blot detection of recombinant HA-StrepII-tagged PvAPRTs in crude extracts from *N. benthamina* leaves probed with anti-HA antibodies. (c) Detection of affinity purified HA-StrepII-tagged PvAPRTs proteins (d) Determination of APRT activity of *N. benthamiana* leaf extracts overexpressing each APRT isoform. (e) Activity purified HA-StrepII-tagged PvAPRTs proteins. Φ control, empty vector; 1, *PvAPRT1; 2, PvAPRT2; 3, PvAPRT4, and 4, PvAPRT5 (** p < 0.005 and *** p < 0.0005).*

(Table 1, and Supplementary Figure S4). Despite the lower affinity found for adenine in APRT1, this isoform was the one with higher V_{max} and catalytic constant, followed by APRT5 that showed higher affinity for adenine but about 7-fold lower V_{max} and catalytic constant values than the APRT1 isoform (Table 1). By contrast, APRT4 showed even lower Vmax and catalytic values than APRT5, whereas lack of activity of isolated PvAPRT2 avoided us for determination of the kinetic characteristics of this isoform.

Table 1. kinetic parameters of PvAPRT isoforms

	Adenine			PRPP		
Protein	**Km (mM)**	**V_{max} (µmoles mg^{-1} min^{-1})**	**K_{cat} (min^{-1})**	**Km (mM)**	**V_{max} (µmoles mg^{-1} min^{-1})**	**K_{cat} (min^{-1})**
APRT1	0.36 ± 0.061	9.8 ± 0.66	0.426	0.94 ± 0.28	11.59 ± 2.9	0.507
APRT2	-	-	-	-	-	-
APRT4	0.071 ± 0.018	0.67 ± 0.04	0.0291	0.23 ± 0.05	0.73 ± 0.052	0.0023
APRT5	0.062 ± 0.032	1.49 ± 0.2	0.0612	0.28 ± 0.037	1.082 ± 0.06	0.0444

APRTs expression and enzyme activity in P. vulgaris tissues

Relative transcript abundance of each *PvAPRT* in various *P. vulgaris* tissues was measured by qRT-PCR in plants grown under nitrogen fixation or fertilized with NO_3^-. Relative expression of *PvPRT2* and *PvAPRT4* was lower in any tissue than those of *PvAPRT1* and *PvAPRT5* genes. *PvAPRT5* was the gene with the highest expression levels in nodules, followed by *PvAPRT1*, that showed the highest expression level in most plant tissues (Figure 3a). Moreover, nitrogen source did not significantly affect the expression level of any of the *PvAPRT* genes. Using the optimized APRT assay, enzyme activity was measured in common bean tissues grown under nitrogen fixation or under NO_3^- fertilization. The assay showed similar APRT activity levels in trifoliate leaves and stems in plants under the two nitrogen conditions and slightly lower activity in the roots of nitrate grown plants than roots of plant under nitrogen fixation. In addition, activity

levels were higher in nodules, roots, stems and pods of nitrogen fixing 28 days-old plants than in the leaves (Figure 3b).

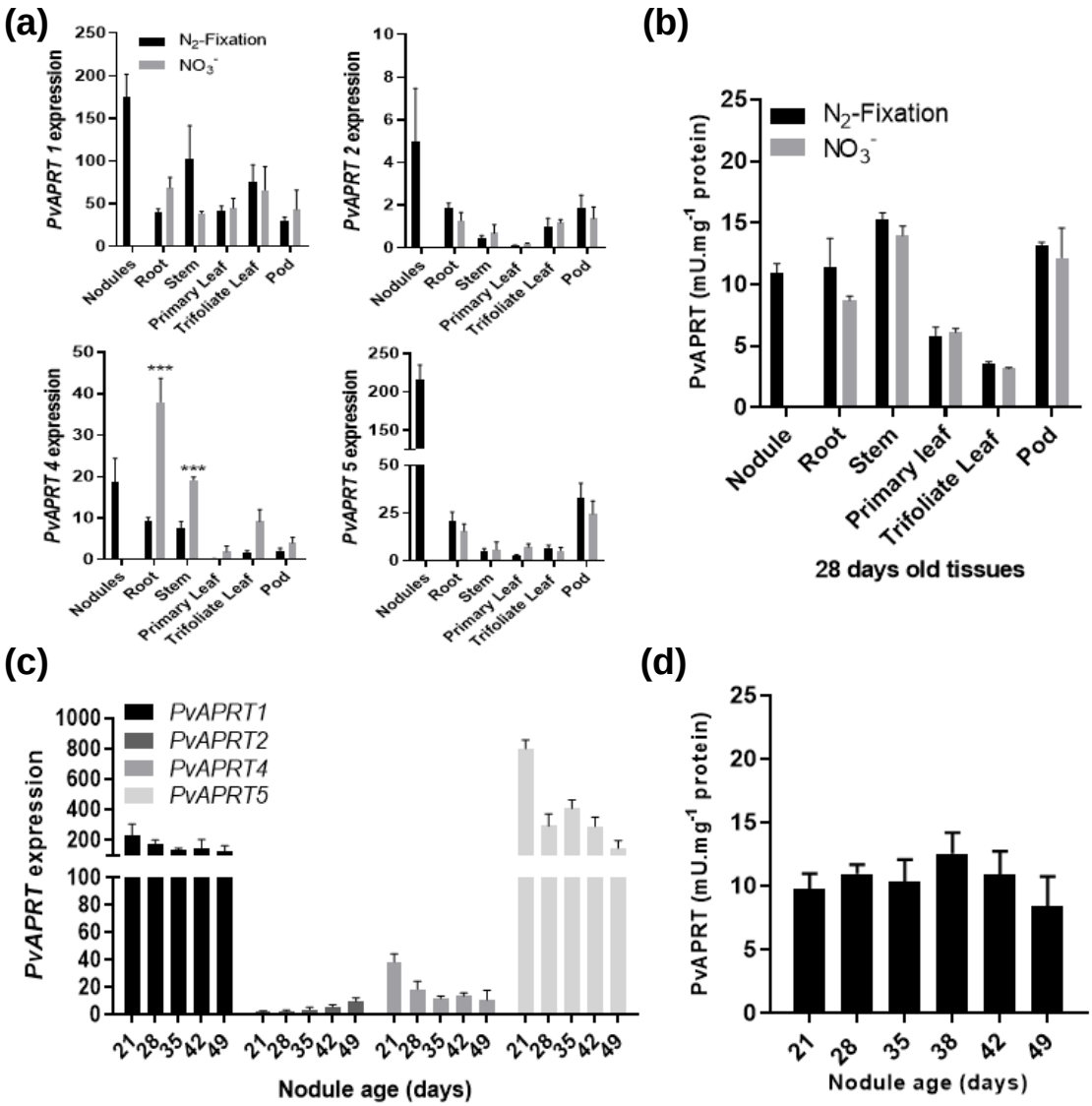

Figure 3. Expression and activity levels of PvAPRTs in *P. vulgaris* tissues. (a) Relative expression levels of *PvAPRT1, PvAPRT2, PvAPRT4* and *PvAPRT5* transcripts determined by qRT-PCR in nodules, roots, stems, primary leaves, 4th trifoliate leaves and pods of 28 days old *P. vulgaris* plants cultivated under nitrogen fixation conditions or nitrate fertilization. (b) Total enzymatic activity in nodules, roots, stems, primary leaf, 4th trifoliate leaves and pods from common bean plants grown for 28 days under N_2-fixation or fertilized with NO_3^- (c) Relative expression of *PvAPRT1, PvAPRT2, PvAPRT4* and *PvAPRT5* genes along nodule development. (d) Total APRT enzymatic activity levels from nodule tissues at several developmental stages. (* $p < 0.05$) (*** $p < 0.0005$).

Relative expression of *PvAPRT* genes in nodules at different developmental stages confirmed that *PvAPRT5* was the gene with the highest expression levels along development of the nodules, followed by *PvAPRT1*. Moreover, the expression of *PvAPRT4* and *PvAPRT5* was highest at early nodule development, decreasing with

nodule ageing, although the *PvAPRT5* expression level was much higher than that of *PvAPRT4*. Relative expression of *PvAPRT1* and *PvAPRT2* genes was kept at constant low levels along nodule ontogeny, thus indicating that *PvAPRT5* is the predominant gene expressed in nodules (Figure 3c). Enzymatic activity was kept at relative constant levels in nodules at different developmental stages (Figure 3d), despite the changes in *PvAPRT4* and *PvAPRT5* gene expression during nodule development. Altogether, expression and activity levels in tissues of plants grown under the two nitrogen sources was similar, although a high expression and activity was surprisingly found in nodules, where *de novo* synthesis of purine nucleotides is highly prevalent.

The *in-silico* analysis of the 1500bp proximal promoter sequences of each *PvAPRT* gene showed several *cis*-regulatory motives related to plant development and a wide variety of motives associated with stress or defence response (Supplementary Figure S5). In the *PvAPRT1* promoter sequence they were eleven MYC and MYB binding site motifs, one involved in drought-inducibility (MBS) and other related with salicylic acid (TCA-element). In addition, they were several motifs related to light responsiveness, such as four Box4-motifs and one of ATCT-motif, AE-box, Gap-box, LAMP-element and MRE, apart from some other related with plant development and senescence, such as one P-box and two ERE, associated with gibberellin and ethylene responses, respectively. A high number of motifs related to the response to different types of stress were also found in the promoter sequence of *PvAPRT2*, although most of them were elements involved in the abscisic acid responsiveness (ABRE). Interestingly, *PvAPRT2* appeared among the upregulated genes in a recent transcriptomic analysis in response to drought stress in common bean (López *et al.*, 2023). *PvAPRT2* had also several motifs associated with light responsiveness and one with auxin-responsiveness (TGA-element). On the other hand, like *PvAPRT1*, most of the motifs associated with stress response in the promoter of *PvAPRT4* gene were MYC and MYB-binding sites, apart from one related with abscisic acid (ABRE) and two with jasmonic acid (CGTCA-motif). Additionally, *PvAPRT4* promoter had 4 sites of ethylene response (ERE), one element involved in zein metabolism (O_2-site) and one P-box gibberellin-responsive element. Finally, the *PvAPRT5* promoter was more like that of *PvAPRT2*, where not only there were MYC and MYB binding sites, but also ABRE cis-acting element, one LTR related as a cis-acting element involved in low-temperature responsiveness, three ERE associated with ethylene responses, four ARE elements, essential for the anaerobic induction and one TCA-element involved in salicylic acid responsiveness

(Supplementary Figure S5). Differences in regulatory motifs in the promoter sequences of the four gene copies suggest that function of these genes may not be totally redundant and a role of the PvAPRTs in several defence and stress responses.

Targeted CRISPR/Cas9 PvAPRT1 and PvAPRT5 gene knock-out.

To elucidate the possible role of APRT proteins in the roots and nodules of common bean plants, downregulation of *PvAPRT1* and *PvAPRT5*, the two APRT genes showing the highest expression levels in these tissues, was achieved using CRISPR/Cas9 gene editing and RNAi silencing. Two gRNA guides targeting first and third and second and third exonic sequences in *PvAPRT1* and *PvAPRT5*, respectively, were generated and introduced into the binary pFGC-pco-Cas9 vector, already expressing the Cas9 nuclease (Figure 4). Expression levels of the targeted genes was chosen to screen the effectivity of gene targeting, since indels generated by repair of Cas9 DNA cuts at the target site would lead to aberrant transcripts, usually prone to degradation (Figure 4 and Supplementary Figure S6).

Transgenic roots with the edition construct targeting *PvAPRT1* showed a significant decrease in their relative transcript abundance compared to that in control roots transformed with the empty vector (Figure 5a, c). Furthermore, these transgenic roots did not show a significant decrease in the expression levels of any of other genes encoding APRTs in common bean, confirming the specificity of the CRISPR/Cas9 targeted mutation. In the same way, in the targeted *PvAPRT5* roots, the relative expression of the *PvAPRT5* was reduced, while the expression of the other three genes encoding APRTs was kept without significant changes (Figure 5b). These results confirmed that CRISPR/Cas9 editing had been done specifically in the targeted sequences from transformed roots.

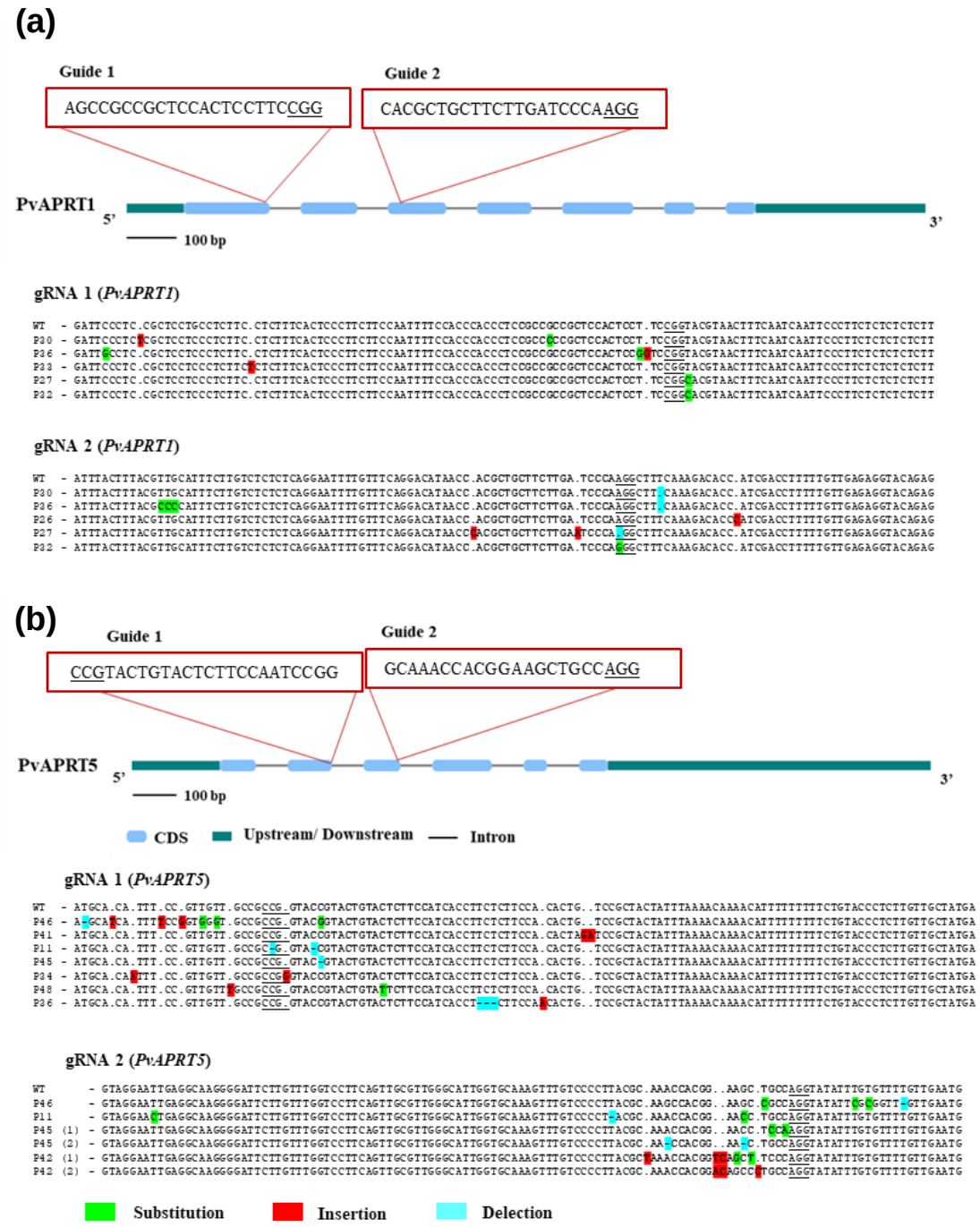

Figure 4. Targeted mutations detected in common bean roots and induced by CRISPR/Cas9 technology. Targeted mutations close to guide 1 (gRNA 1) and guide 2 (gRNA 2) regions in the (a) *PvAPRT1* and (b) *PvAPRT5* gene. Target sequences are highlighted in bold and PAM sequences are underlined. The code P (number) corresponds to the selected plant and substitutions, insertions and deletions are underlined in green, red, and blue, respectively. Number in parentheses represents different cloned DNA sequences.

In addition, total APRT activity with adenine and PRPP as substrates was lower in the transgenic roots than in the empty vector transformed ones. The decrease in adenine salvage activity was higher and more significant in transgenic roots with mutated *PvAPRT1* genomic sequence than in those with targeted *PvAPRT5*. Specifically, targeted APRT1 showed a 47% activity with adenine as substrate of that in controls, whereas it was 70.4% in the APRT5 transgenic roots (Figure 5c, d).

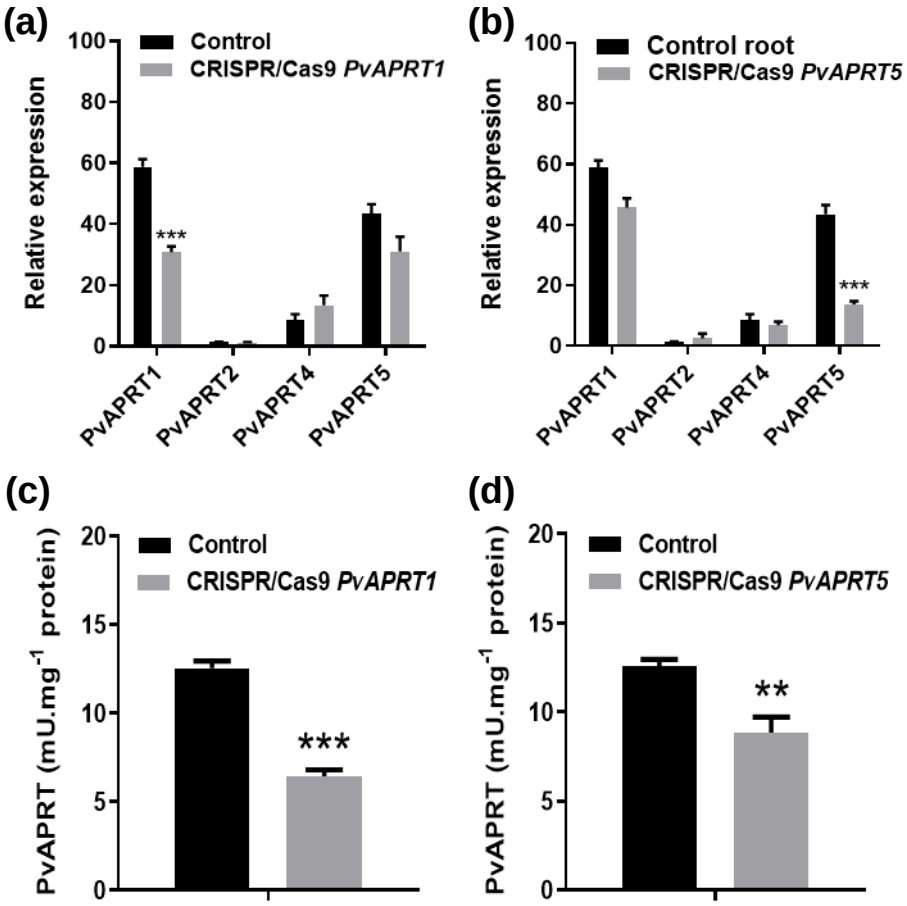

Figure 5. Relative gene expression and enzymatic activity of PvAPRT in CRISPR/Cas9 transformed hairy roots. (a) Relative expression of *PvAPRT1, PvAPRT2, PvAPRT4* and *PvAPRT5* genes in *PvAPRT1* targeted transgenic roots (b) Relative expression in *PvAPRT5* CRISPR/Cas9 targeted hairy roots. (c) Total APRT enzymatic activity in CRISPR/Cas9 *PvAPRT1* targeted roots (d) *PvAPRT5* targeted roots (*$p < 0.05$ and ** $p < 0.005$ and *** $p < 0.0005$).

To further validate the results obtained using the CRISPR/Cas9 technology, DNA extraction was performed from several hairy roots with lower expression levels of the *APRT 1* or *5* genes than that in control roots transformed with empty vector. Gene sequence analysis of the regions near the gRNA guides targeted sequences revealed the presence of different mutations in these regions. Among them there were several short

insertions, deletions, and substitutions, some causing premature terminations of the *PvAPRT1* and *PvAPRT5* transcripts (Figure 4).

Additionally, transgenic roots were also obtained with downregulation of the *PvAPRT1* and *PvAPRT5* genes using RNA interference (RNAi). However, RNAi mediated silencing was found to be less specific and less efficient than CRISPR/Cas9 editing. The *PvAPRT5* siRNA did not only reduced the expression level of *PvAPRT5*, but it also significantly decreased *PvAPRT1* gene expression (Supplementary Figure S7a, b). Moreover, decrease in total APRT activity was lower in the RNAi roots than in the CRISPR/Cas9 targeted ones (Supplementary Figure S7c, d). Therefore, CRISPR/Cas9 targeted *PvAPRT1* and *PvAPRT5* were used for subsequent functional analysis.

Metabolomic analysis from edited PvAPRT1 and PvAPRT5 common bean plants

To further investigate APRT1 and 5 function in common bean, GC/MS, and LC/MS metabolomic analysis, to find changes in primary and secondary metabolites, was performed on the extracts from CRISPR/Cas9 *PvAPRT1* and *PvAPRT5* targeted roots. The analysis, performed using six independent hairy roots with downregulated levels of the respective transcripts, revealed that the roots with the *PvAPRT1* and *PvAPRT5* targeted genes exhibited differential quantitative and qualitative metabolite changes, suggesting differences in the functions for each of the two proteins (Figure 6a; Supplementary Table S4). There was a total of 28 metabolites exhibiting significant concentration differences in roots with mutated *PvAPRT1* in comparison to controls, and 39 metabolites in those targeting *PvAPRT5* (Figure 6b).

The transgenic roots with mutated *PvAPRT1* showed significant accumulation of adenine. However, adenine accumulation was not found in the targeted *PvAPRT5* roots (Figure 6a), indicating that APRT1 is the preferred isoform dedicated to adenine salvage in common bean roots. Moreover, increments in the relative concentration of purine derivatives such as inosine and adenosine monophosphate were found in the targeted roots. The increases in inosine were lower in the targeted *PvAPRT1* than in *PvAPRT5* targeted roots. Moreover, *PvAPRT1* and *PvAPRT5* mutant roots exhibited accumulation of the cytokinin hormone N6-benzyl adenine, but accumulation was only significant in the roots with reduced *PvAPRT5* (Supplementary Table S4). Hairy roots with reduced expression of the two *PvAPRT* genes also showed differential levels of several amino acids, alanine, glutamic acid and aspartylphenylalanine accumulated in transgenic *PvAPRT1*, whereas, serine, isoleucine, isoleucylproline, and glutamylmethionine

accumulated in the targeted *PvAPRT5* but not in *PvAPRT1* ones. Transgenic roots also showed different increased levels of carbohydrates (raffinose, erythrose, mannose), and metabolites involved in the glycolysis and the tricarboxylic acid cycle (glucose-6-phosphate, 2-oxo-glutaric acid, isocitric acid, pyruvic acid, succinic acid and threonic acid) (Supplementary Table S4). Moreover, stress related phytohormones, as salicylic

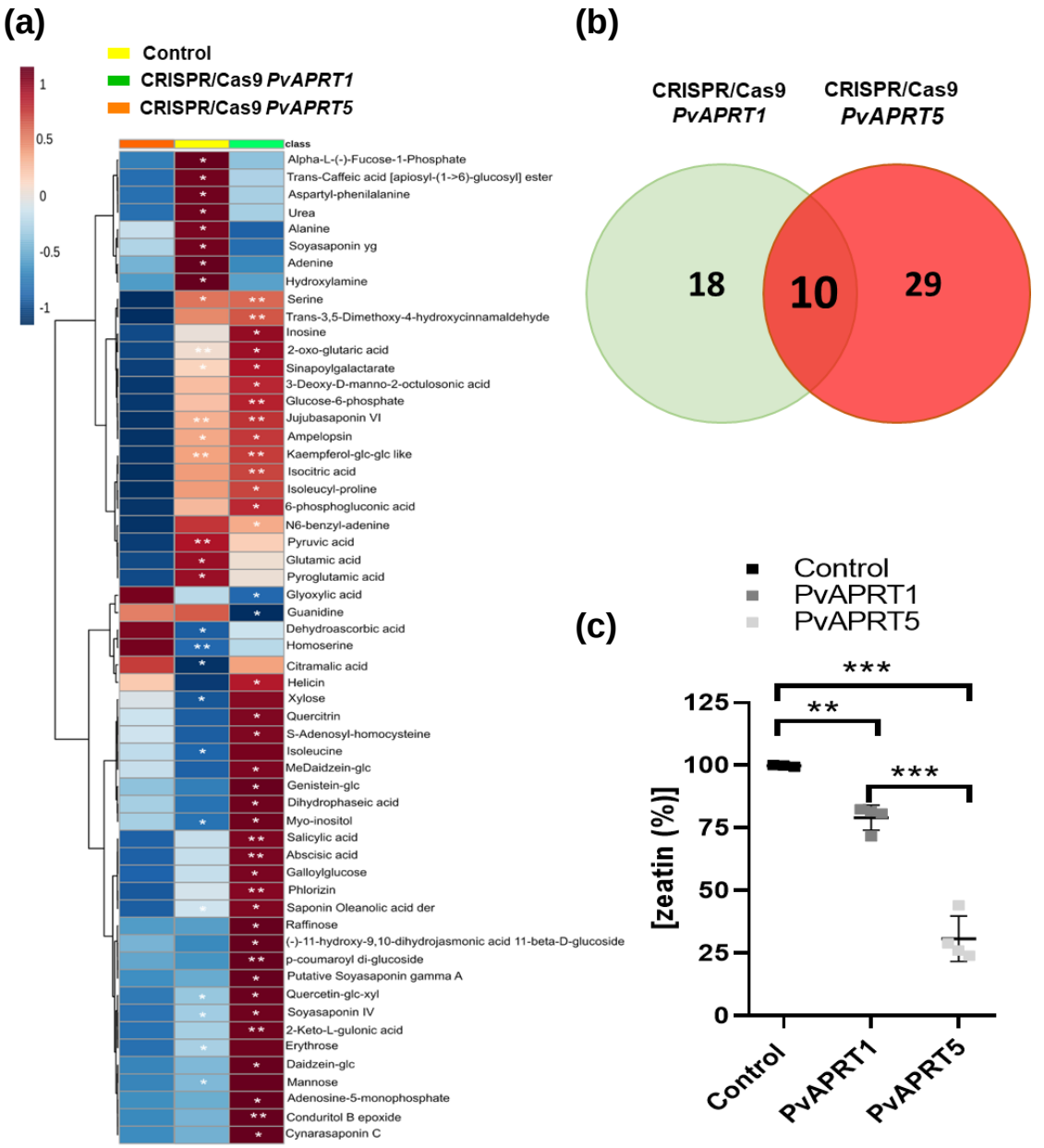

Figure 6. Metabolomic analysis from transgenic roots. (a) Heat map of the primary and secondary metabolites with significant differences ($p < 0.05$) after comparison of CRISPR/Cas 9 hairy roots (*PvAPRT1* or *PvAPRT5*) with control tissues. (b) Venn diagrams with the total number of metabolites with significant differences ($p < 0.05$) when hairy root with *PvAPRT1* (RED) and *PvAPRT5* (GREEN) genes downregulated, are compared with control tissues. (c) APRT activity upon zeatin nucleobase. Zeatin concentration decay determined after enzymatic assay with purified PvAPRT1 and PvAPRT5. *(* $p < 0.05$; ** $p < 0.005$* and **** $p < 0.0005$*).

acid and abscisic acid, as well as a greater number of flavonoids and saponins significantly accumulated in transgenic *PvAPRT5*-targeted roots (Supplementary Table S4).

Since metabolomic analysis strongly suggested that the two APRTs could have some functional specialization, activity catalysing the conversion of zeatin nucleobase to zeatin nucleotide was assayed using the two purified enzymes. After the reaction samples were lyophilized and submitted to metabolite analysis. Results in Figure 6c revealed that APRT1 transformed less than 30 % of zeatin, whereas the APRT5 isoenzyme was able to transform over 75% of the initial zeatin nucleobase substrate. These data, thus, indicate that although both isoenzymes could transform zeatin nucleobase (active cytokinin) to the nucleotide (inactive), APRT5 exhibits considerably higher activity on this substrate than APRT1.

Physiological analysis of APRTs transgenic hairy roots

Analysis of individual hairy roots that developed from the transformation wounds revealed that approximately 65% and 80% of the roots showed lower *PvAPRT1* and *PvAPRT5* expression levels, respectively, than the mean of the control ones, transformed with the empty vector (Figure 7a). The presence of hairy roots with control expression levels was not surprising since the hairy roots can be induced by *R. rhizogenes* infection both in transformed and untransformed cells at the wounding site. Moreover, the number of nodules per plant was similar in control as in the *PvAPRT*s targeted roots (Figure 7b), and nodules appeared both in roots with downregulated expression of the *PvAPRT* genes (silenced) or controls (non-silenced). However, the number of *PvAPRT*s silenced transgenic hairy roots without nodules was higher than of the nodulated transgenic roots (Figure 7c). Accordingly, nodules from PvAPRT1 and PvAPRT5 hairy roots showed expression levels of the two genes like that in control ones (Supplementary Figure S8a). Consequently, APRT activity was also at control levels in the nodules of the CRISPR/Cas-APRTs targeted plants (Supplementary Figure S8).

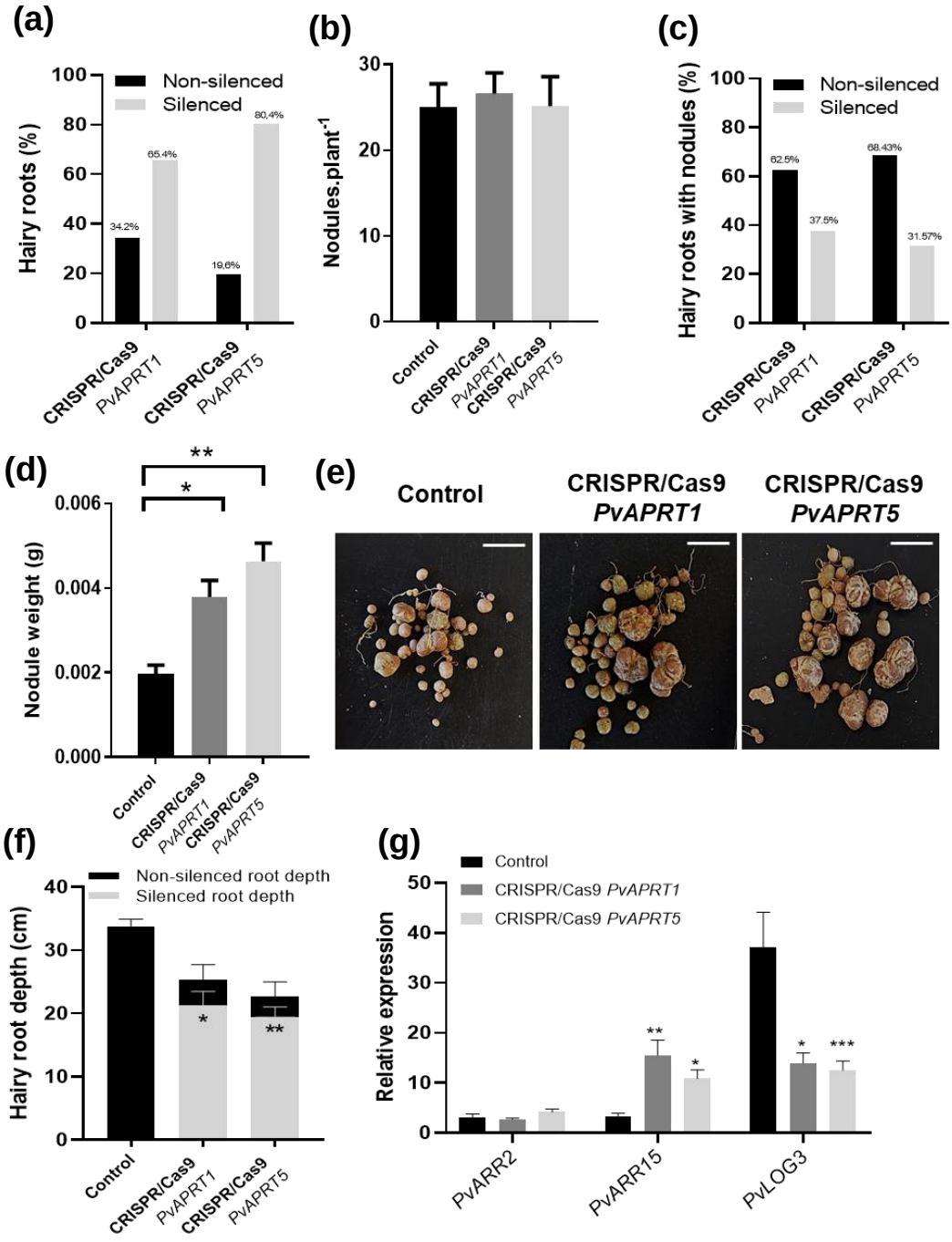

Figure 7. Physiological analysis from transgenic root and nodule tissues. (a) Percentage of CRISPR/Cas9 *PvAPRT1* and *PvAPRT5* hairy roots with downregulated (silenced) *PvAPRT1* and *PvAPRT5* expression levels. (b) Nodule number in CRISPR/Cas9 *PvAPRT1* and *PvAPRT5* targeted hairy roots. (c) Percentage of CRISPR/Cas9 *PvAPRT1* and *PvAPRT5* targeted hairy roots with and without nodules. (d) Nodule weight from control and transgenic hairy roots. (e) Size of nodules from transgenic root of *P. vulgaris* with CRISPR/Cas9 *PvAPRT1* and *PvAPRT5* downregulated genes. White line 0.25 cm. (f) Root depth from control and transgenic *P. vulgaris* roots. (g) Relative expression of genes related with cytokinin metabolism in control and silenced *PvAPRT1* and *PvAPRT5* hairy roots (*$p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$).

In contrast, the size and biomass of the nodules in *PvAPRT1* and *5* targeted roots were significantly larger than that of control hairy roots transformed with the empty vector (Figure 7d). Increment in nodules size where more significant in the transgenic roots with the targeted PvAPRT5 gene and to a lesser extent in *PvAPRT1* gene edited roots (Figure 7d and e). Interestingly, root length in those roots showing downregulation of the *PvAPRT1* and *5* was significantly lower than that of the hairy roots in the untargeted controls. This result was more pronounced in roots with the *PvAPRT5* downregulated gene than in those with the targeted *PvAPRT1* (Figure 7f).

Effects on nodule size and root length, besides the higher accumulation of cytokinin in the targeted *PvAPRT5* transgenic roots, prompt us to analyse the expression of genes related with the cytokinin activation and signalling. Interestingly, expression of cytokinin-activated signalling A-Type ARRs, *Arabidopsis* Response Regulator (*PvARR15*), was induced in both *PvAPRT1* and *PvAPRT5* transgenic roots. Instead, there were no changes in the expression of *PvARR2* (Figure 7g). This difference is not surprising since ARR2 is a B-Type regulator, and these two groups have been shown to have opposite effects on cytokinin activating signalling (Hwang and Sheen, 2001; Kiba *et al.*, 2002; Müller and Sheen, 2007). On the other hand, the expression of the LONELY GUY (LOG) gene, coding a cytokinin riboside phosphoribohydrolase that catalyzes the release of free-base, active cytokinin, from cytokinin ribotides (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009), was reduced in the transgenic roots with mutated *PvAPRT1* and *PvAPRT5* (Figure 7g). The decrease in *PvLOG* expression was higher and most significant in the *PvAPRT5* mutant than the *PvAPRT1* mutant, thus supporting the previous results of a higher implication of APRT5 in cytokinin inactivation than that of APRT1.

Discussion

Adenine phosphoribosyltransferase (APRT) catalyzes the conversion of adenine base to nucleotide (Figure 1a), which is the main AMP supply in animals and many organisms (Henderson and Paterson, 2014; Kornberg *et al.*, 1955). In plants, purine nucleotides are obtained by the *de novo* synthesis in chloroplast, but the salvage of adenine does also have relevant functions in regulation of plant growth and development. Adenine bases and nucleosides are produced by turnover of nucleotides and nucleic acids, but also from the S-adenosyl-L-methionine (SAM) cycle in ethylene, nicotianamine and polyamines biosynthesis pathways, and by the activity of methyltransferases as in caffeine and

glycine betaine production (Ashihara *et al.,* 2018). Besides adenine salvage, the plant APRT enzyme also plays a crucial role in cytokinin metabolism (Allen *et al.*, 2002; Zhang *et al.,* 2013). Nonetheless, despite its functional relevance, characterization of purine salvage enzymes is scarce in plants. In ureidic legumes, as common bean, *de novo* synthesized purine nucleotides in nodules are the precursors ureides which are the main nitrogen transport molecules and are also important in stress responses (Alamillo *et al.,* 2010). Therefore, understanding the role of purine salvage by the APRT protein could be relevant not only because its effects on plant development but also on ureide synthesis and tolerance to stress.

In the *P. vulgaris* genome there are four genes that encode the APRT protein (*PvAPRT1, PvAPRT2, PvAPRT4* and *PvAPRT5*), named according to their homologues in the five *Arabidopsis* APRTs, whereas there is not a homologous to APRT3 in the common bean genome (Allen *et al.,* 2002; Men *et al.,* 2021). This work shows that members of the common bean APRT family are classified into two different clusters, one containing the PvAPRT1 and PvAPRT4 isoforms, and the second containing PvAPRT2 and PvAPRT5 (Figure 1b). The PvAPRT1 and 4 proteins contain a N-terminal signal peptide for chloroplast targeting, that is absent in the APRT2 and 5 of the second cluster (Figure 1c). Chloroplast location of APRT 1 and 4 and cytosolic location of APRT 2 and 5 from common bean was confirmed by YFP-tagging of the four proteins (Figure 2a). Chloroplast location of PvAPRT1 and 4 differed to early data from *Arabidopsis* APRTs, from which cytosolic location was reported for AtAPT1, 2 and 3 (Allen *et al*., 2002). Nonetheless, a possible chloroplast targeting peptide is present in *Arabidopsis* APRT1 isoform (Figure1b), and APRT1 has been found in a chloroplast proteome analysis (Zybailov *et al.,* 2008), indicating that the APT1 from *Arabidopsis* could be also located in chloroplast. Chloroplast targeting sequence is also present in the APRT1 homologues from soybean, although not in *Medicago* and algae APRTs (Figure 1c). Dual presence of APRT proteins in chloroplast and cytosol is not surprising, since PRPP synthetase (EC 2.7.6.1, ATP: D-ribose-5-phosphate diphosphotransferase), which generates the PRPP substrate for APRT activity, is present in both the chloroplast and cytosol, but it might suggest a possible differential functionality of the different APRT isoforms (Andriotis and Smith, 2019; Ashihara, 2016).

Expression of recombinant tagged proteins, demonstrated that the four genes encode mature proteins of the expected molecular mass (Figure 2b; Supplementary Figure S3). Moreover, recombinant PvAPRT1, PvAPRT4 and PvAPRT5 isoforms were active using

adenine as substrate, whereas we could not detect activity of PvAPRT2. The kinetic parameters of affinity purified proteins showed that although PvAPRT1 had the lowest affinity for adenine, it was the isoform with the highest Vmax, whereas APRT4 and 5 exhibited higher substrate affinity, but lower Vmax. Similar opposite trend among Km and Vmax for adenine was reported for the recombinant *Arabidopsis* APRTs 1, 2 and 3, although Km values for common bean APRTs where higher than those in *Arabidopsis* (Allen *et al.*, 2002), perhaps reflecting higher interference with the substrate binding pocket of the HA-Strep tagging sequence compared to the 6xHis in *Arabidopsis* recombinant proteins. Using the recombinant *Arabidopsis* proteins Zhang *et al.*, (2013) showed that AtAPT1 had the highest activity with adenine, whereas APT5 showed comparable activity to APT1 in zeatin conversion, but lower activity than APT1 when using adenine. Moreover, APT4 exhibited about half the activity of APT1, and the purified AtAPT2 failed to show any catalytic activity. As in *Arabidopsis*, APRT1 isoform from common bean has the highest potential metabolic contribution towards adenine, estimated as Vmax/Km, although APRT5 isoform also has higher potential than PvAPRT4 and PvAPRT2 was also inactive as it was the *Arabidopsis* APT2 (Table 1; Supplementary Figure S4).

Expression analysis of the *PvAPRT*s genes in plants grown under nitrogen fixation or fertilized with nitrate (NO_3^-) showed that transcripts levels of *PvAPRT1*, *2* and *5* were independent of the nitrogen sources. Only *PvAPRT4* showed higher expression in roots and stems of plants with nitrate than in those grown under nitrogen fixation (Figure 3a), although its expression was lower than that of *PvAPRT1* and *5*. The source of nitrogen has been shown to highly influence purine nucleotide metabolism in common bean. Under nitrate fertilization activity of glutamine phosphoribosyl aminotransferase (PvPRAT), first enzyme in the *de novo* synthesis of purine nucleotides is inhibited (Coleto *et al.*, 2016). Therefore, while NO_3^- reduces the expression of genes of the *de novo* synthesis pathway, it increases the expression of at least one APRT coding gene. However, APRT activity remained at constant levels in tissues with the two nitrogen sources (Figure 3b). Therefore, it seems that salvage of adenine to purine nucleotide AMP is maintained despite the activity of *de novo* synthesis. This is not surprising since the salvage activity not only helps to generate AMP, but also to remove adenine and adenosine that could have severe adverse consequences in plant development (Witte and Herde, 2020). Mutation of the main *Arabidopsis* APRT, APT1 results in male sterility (Gaillard *et al.*, 1998), and APRT downregulation increases oxidative stress resistance

(Sukrong *et al.*, 2012).

We found that besides expression in roots, stems and young pods, genes encoding the APRT protein in common bean were highly expressed in nodules (Figure 3a, c). Roots, stems and young pods contain meristematic tissues requiring high levels of nucleotides to sustain cell division. Moreover, in these meristematic tissues regulation of cytokinins levels is important (Dewitte *et al.*, 1999; McAtee *et al.*, 2013; Mok and Mok, 1994). Analysis of possible cis-acting motifs in the promoter of common bean APRT coding genes showed several motifs related to phytohormones and response to stress (Supplementary Figure S5). In addition, plant APRT protein not only acts in the conversion of adenine to AMP, but also catalyses the conversion of cytokinin nucleobases (active) to cytokinin ribotides (inactive) (Ashihara *et al.*, 2018; Lee and Moffatt, 1993; Zhang *et al.*, 2013). Therefore, the high expression of PvAPRTs genes in these tissues could be related to a possible regulatory role of cytokinin activity, instead of or besides, the adenine salvage for AMP synthesis.

In nodules of ureidic legumes, *de novo* synthesis of purine nucleotide pathway is highly induced (Atkins and Smith, 2000; Atkins, 1991; Coleto *et al.*, 2016) and, apparently, the salvage route in which the APRT protein participates might be less necessary. However, high expression *PvAPRT* genes, especially *PvAPRT5*, was found in nodules. In addition, *PvAPRT4* and *5* genes displayed their highest expression levels during early nodule development. At the beginning of nodule development, the main protein regulating *de novo* synthesis of nucleotides, PRAT, still does not have its highest level of activity (Coleto *et al.*, 2016). Therefore, it is possible that at early stages of nodule development the salvage purine enzymes would be required to synthesize purine nucleotides. Nonetheless, high expression of genes at these earlier stages were not followed by elevated levels of APRT activity, at least with adenine as substrate. Noteworthy, APRT1 was the isoform with the highest activity using adenine as substrate, and the expression of this gene did not change during the ontogeny of the nodules. Thus, although PvAPRT1 could be involved in adenine salvage in nodules, the highest expression levels of PvAPRT5 at early nodule development suggest a more specific function of this isoform during nodule development.

Gene editing by CRISPR/Cas9 technology allows the precise targeting of sequences (Zhang *et al.*, 2019, 2017) and, together with the hairy roots transformation, have been exploited to dissect the function of gene family members in legume roots (Alamillo *et al.*, 2023; Voß *et al.*, 2022). In this study, CRISPR/Cas9 gene editing has been applied

to generate mutation of *PvAPRT1* and *PvAPRT5*, the two genes more highly expressed in common bean roots and nodules. Targeted mutations of *PvAPRT1* and *PvAPRT5* rendered specific downregulation of the corresponding proteins, whereas posttranscriptional RNAi silencing of these two genes also altered the expression levels of other *PvAPRT* transcripts, confirming the higher specificity of CRISPR/Cas9 gene editing compared to silencing by RNAi (Figure 5; Supplementary Figure S8).

Metabolomic analysis revealed that PvAPRT1 knockout roots significantly accumulated more adenine than controls (Figure 6a). This coincided with the significant (up to 50%) decrease in APRT enzyme activity using adenine as substrate (Figure 5c). Although the reduction in the expression obtained by the CRISPR/Cas9 editing of *PvAPRT5* was higher than for the targeted *PvAPRT1*, the accumulation of adenine was very low in the mutant *PvAPRT5* roots. In addition, the reduction in activity with adenine in *PvAPRT5* edited hairy roots was lower than in mutant *PvAPRT1* tissues. It is possible that in *PvAPRT5* mutants the salvage of adenine could be compensated by the APRT1 isoform, thus limiting the effects of APRT5 inhibition. Nonetheless, these results confirm a higher contribution of APRT1 to adenine salvage in the generation of AMP than that of the APRT5 isoform. Moreover, reduction in the levels of the two isoforms led to differential metabolomic changes in the two mutant roots groups, indicating that APRT1 and APRT5 proteins could play different functions in plants.

Early studies in *Arabidopsis* APT1 isoform indicated a role in the conversion of active cytokinin nucleobases to inactive cytokinin nucleotides (Moffatt et al., 1991; Allen *et al.*, 2002). Later, inactivation of cytokinins by APT1catalyzed phosphoribosylation was demonstrated (Zhang *et al.*, 2013). Our metabolomic analysis revealed that transgenic roots with targeted *PvAPRT5* gene significantly accumulated more N6-benzyladenine and zeatin-glucoside cytokinins than control roots (Figure 6 and Supplementary Table S4). Moreover, activity assay using purified enzymes and zeatin as substrate demonstrated that PvAPRT5 had a much higher activity upon the cytokinin substrate than PvAPRT1 (Figure 6c), confirming that, although both isoforms can act on adenine and cytokinin as substrates, they do so with differential affinities. Differences in metabolomic profiles in *PvAPRT1* and *PvAPRT5* mutant roots agrees with functional specialization of the two isoforms. Besides adenine, mutant APRT1 roots also accumulated several amino acids, hydroxylamine, urea and caffeic acid, whereas mutant *PvAPRT5* roots accumulated stress related compounds, including abscisic acid and salicylic acid, and several secondary metabolites such as flavonoids (quercetin),

isoflavones (genistein), glycosides (saponins), as well as carbohydrates, as raffinose, mannose and erythrose. On the other hand, mutant *PvAPRT5* roots accumulated AMP and inosine, whereas the level of adenine was as low as in controls (Figure 6a). AMP and inosine in the mutant roots could result from higher *de novo* purine nucleotide synthesis in the larger nodules of these roots compared to control ones, or by salvage of adenine to AMP, most likely carried out by APRT1 in APRT5 mutant roots. Nonetheless, it would be important to analyse the functional implication of the lower expressed APRT2 and 4 isoforms to determine whether they fulfil specific functions or are redundant to their closest homologues.

Several works have shown that regulation of cytokinin levels is essential for proper nodulation (Frugier *et al.,* 2008). Gain-of-function of histidine kinase 1 (LHK1), related to *Arabidopsis* cytokinin receptor, promoted nodule primordium in absence of *rhizobia* in *Lotus japonicus* (Bauer *et al.,* 1996; Tirichine *et al.,* 2007). By contrast, *L. japonicus* mutants for the cytokinin oxidase/dehydrogenase 3 (CKX3) showed accumulation of active cytokinins and inhibition of nodulation (Reid *et al.,* 2016). In our work, common bean plants with transgenic roots with targeted *PvAPRT1* and *PvAPRT5* genes grown under N_2 fixation conditions showed that a high percentage of the mutant roots with reduced expression of APRTs did not form nodules (Figure 7c). On the other side, nodules formed in the roots of plants with targeted *PvAPRT1* and *PvAPRT5* were larger than those in the control hairy roots, and the effect was more pronounced in the mutant *PvAPRT5* (Figure 7d, e). This, together with the expression patterns of the *PvAPRT5* gene at early stages of nodule development, suggest that this isoform has a role in nodule development through the regulation of cytokinin levels.

Cytokinins control cell division and lateral root formation and low levels of this growth regulator are required for appropriate root growth and development (Skoog and Miller, 1957). *Arabidopsis* APT1 mutants that accumulated cytokinin N-glucosides showed root growth inhibition (Moffatt *et al.,* 1991; Zhang *et al.,* 2013). Our results showed that transgenic *PvAPRT1* roots, and more significantly *PvAPRT5* ones, had a shorter roots phenotype than control roots (Figure 7f). The effects caused by cytokinins in roots are in part mediated by ethylene signalling and the interaction of cytokinins with auxins (Cary *et al.,* 1995; Ruzicka *et al.,* 2009). High cytokinins levels alters the expression of PIN genes and inhibits the auxin gradient in *Arabidopsis* (Laplaze *et al.,* 2007). The metabolomic analysis showed here revealed a reduction in the amount of auxin indole acetic acid (IAA) in the transgenic roots (Supplementary Table S4). This effect is in

keeping with the higher accumulation of cytokinins and would explain the reduction in root length found in PvAPRT mutant roots.

The changes of expression of the cytokinin response regulators (ARRs) and cytokinin activation lonely guy (LOG) genes in the transgenic APRTs roots (Figure 6g) further corroborate that effects in nodule size and inhibition in root length might be related to the accumulation of cytokinins in these roots. ARRs are a large family of the cytokinin signal transduction, classified into A-Type ARRs, that negatively regulate cytokinin signalling, and B-Type ARR, that are transcription activators of cytokinin response (Hwang and Sheen, 2001; Kiba *et al.*, 2002; Müller and Sheen, 2007). In the targeted APRTs roots, expression of B-type ARR2 was unaltered, whereas expression of the A-Type ARR15 was induced, agreeing with their role as negative regulators to limit the effects of cytokinin accumulation in these roots. On the contrary, expression of the cytokinin activating LOG was reduced in the two transgenic roots. The LOG enzyme catalyzes direct activation of cytokinins (Kuroha et al., 2009), in an opposite reaction to that of APRT, therefore, a reduction of LOG expression is expected in conditions of limited inactivating cytokinin by APRT activity, as in the transgenic APRT hairy roots.

In summary, CRISPR editing coupled to analysis of metabolite changes in the mutant has proven that, although PvAPRT1 and PvAPRT 5 have partially redundant functions catalysing the conversion of adenine to AMP and the inactivation of cytokinins, the two isoforms also show some degree of specialization, with a higher involvement of APRT1 in adenine salvage whereas APRT5 has higher role in regulating cytokinin activity and root and nodule development *in vivo*. Our results also point to an unexplored role of APRTs in several stress responses.

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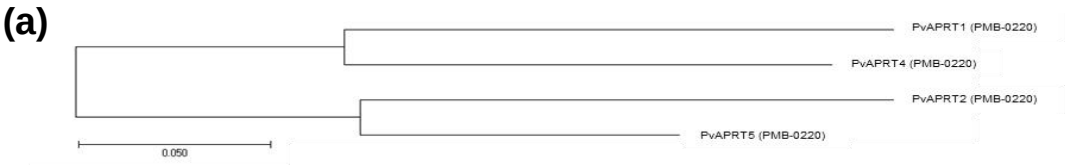
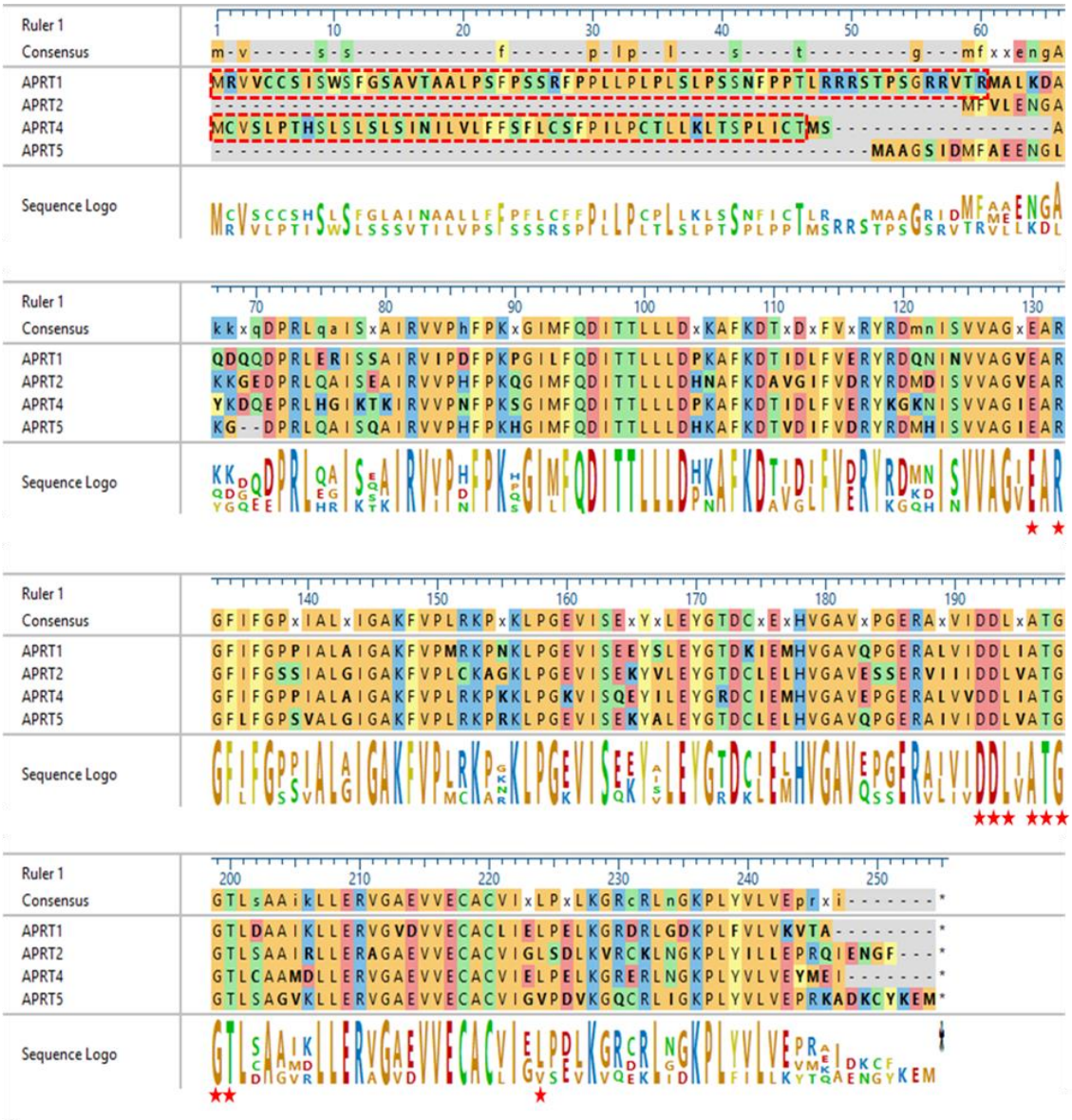
Supplementary material

(a)

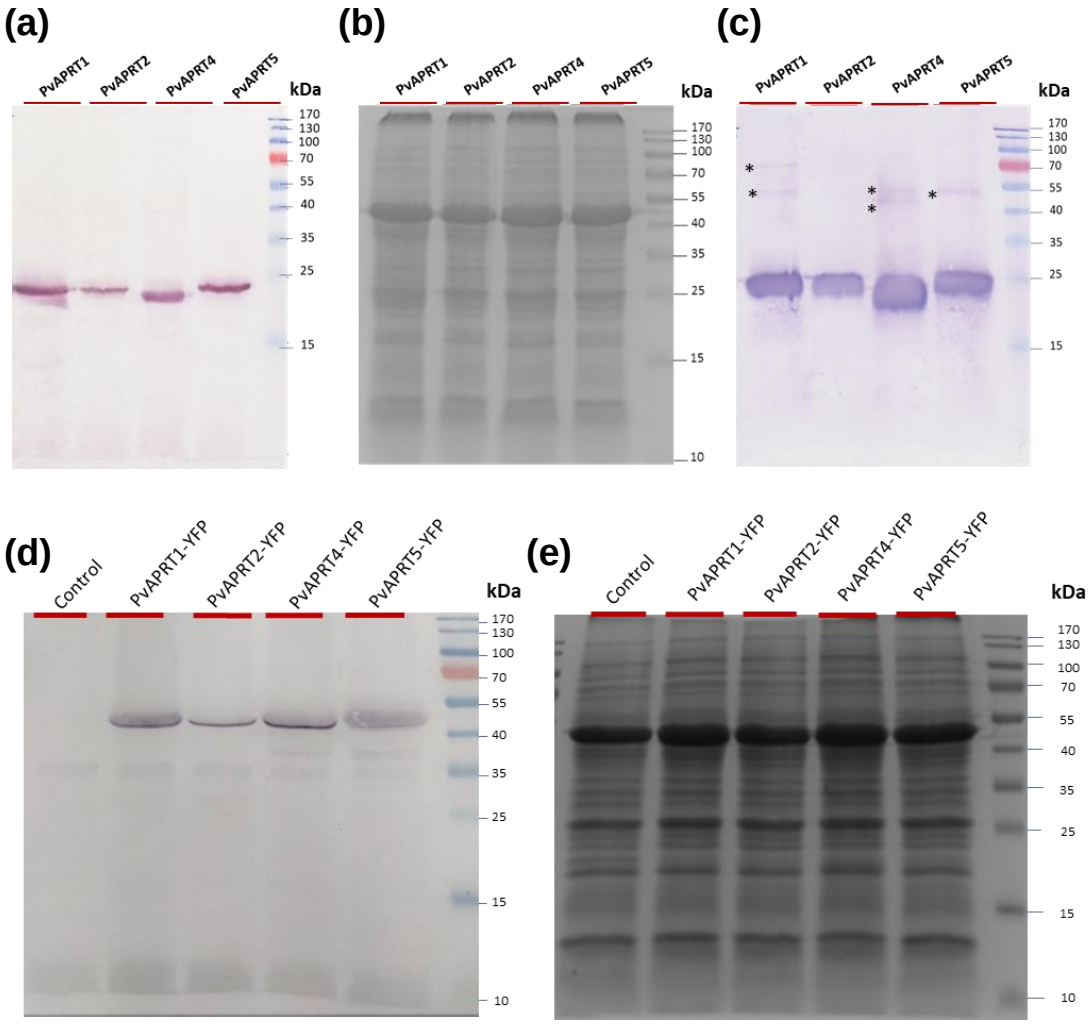


(b)

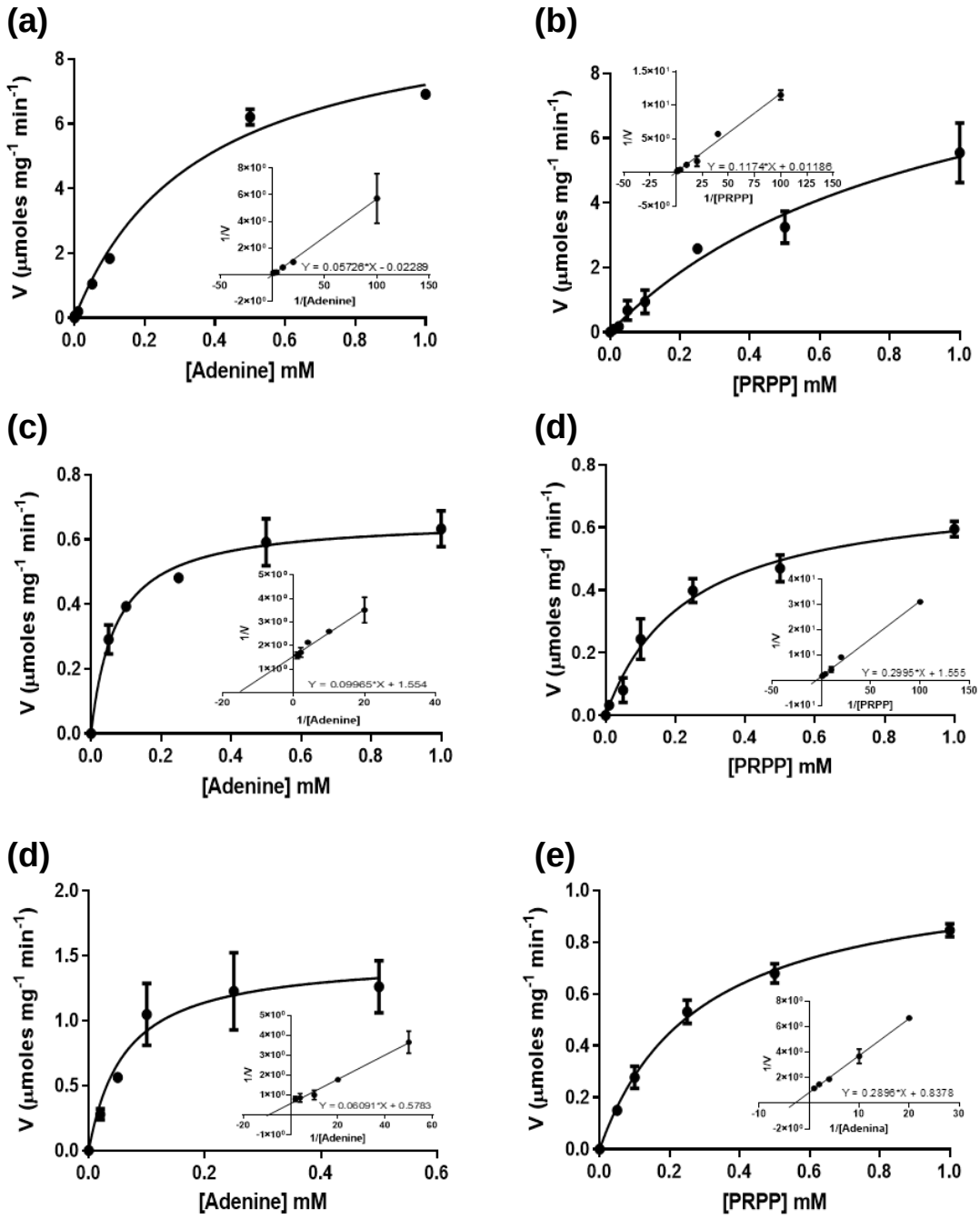


Supplementary Figure S1. Phylogenetic distances of APRTs proteins. (a) Phylogenetic analysis of the four APRT proteins from *P. vulgaris* cv. Great Northern "Matterhorn" (PMB-0220) inferred using the Neighbour-Joining method and MEGA 11 software. (b) Alignment of the four peptide sequences from APRT proteins of *P. vulgaris* (PMB-0220). Inferred signal peptide is indicated by red box. Red stars denote invariable residues at the active site.

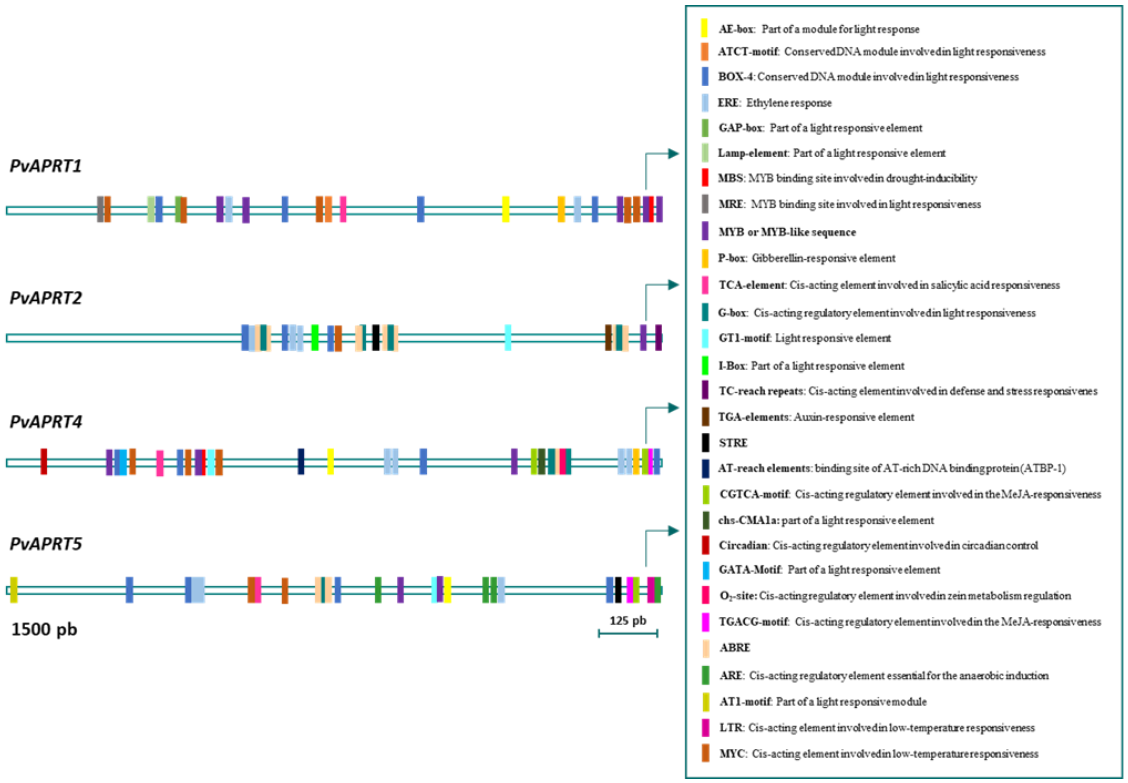
Supplementary Figure S2. Phylogenetic distances of APRTs proteins from different *P. vulgaris* genotypes. (a) Phylogenetic analysis of the four APRT proteins from *P. vulgaris* Great Northern "Matterhorn" (PMB-0220), and from two other genotypes available in Phytozome 13, inferred using the Neighbour-Joining method and MEGA 11 software. (b) Alignment of the peptide sequences of the four APRT isoforms present in the three common bean genotypes performed with Clustal omega software. The green and red boxes represent the signal peptide and invariable residues at the active site, respectively.



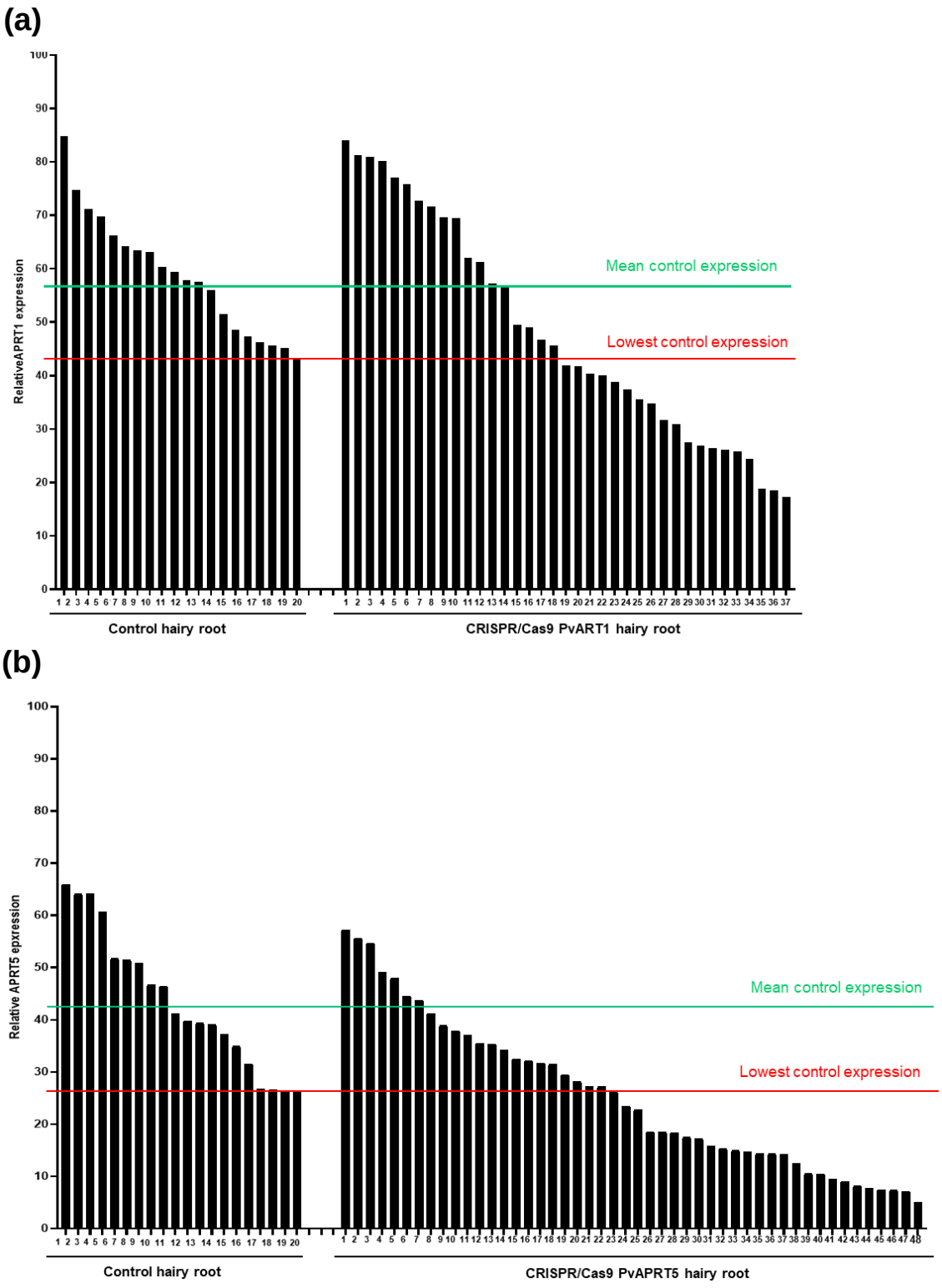
Supplementary Figure S3. Overexpression of HA-Strep-tagged and YFP-tagged PvAPRTs protein in *Nicotiana benthamiana* leaves. (a) Western-blot analysis of HA-Strep-tagged PvAPRTs from APRTs overexpressing *N. benthamiana* leaf extracts, probed with anti-HA monoclonal antibodies. (b) Coomassie staining of leaf extract proteins. (c) Western-blot analysis of recombinant PvAPRTs protein after affinity purification by binding to Srep-Tactin Sepharose. (*) indicates possible dimers and trimers of PvAPRT proteins. (d) Western-blot analysis of YFP-PvAPRTs probed with anti-GFP monoclonal antibodies. (e) Coomassie staining of protein from YFP-PvAPRTs *N. benthamiana* of leaf extracts. PXCSHA-Strep and PXC-YFP vectors were a gift from Prof. C-P Witte (Germany).



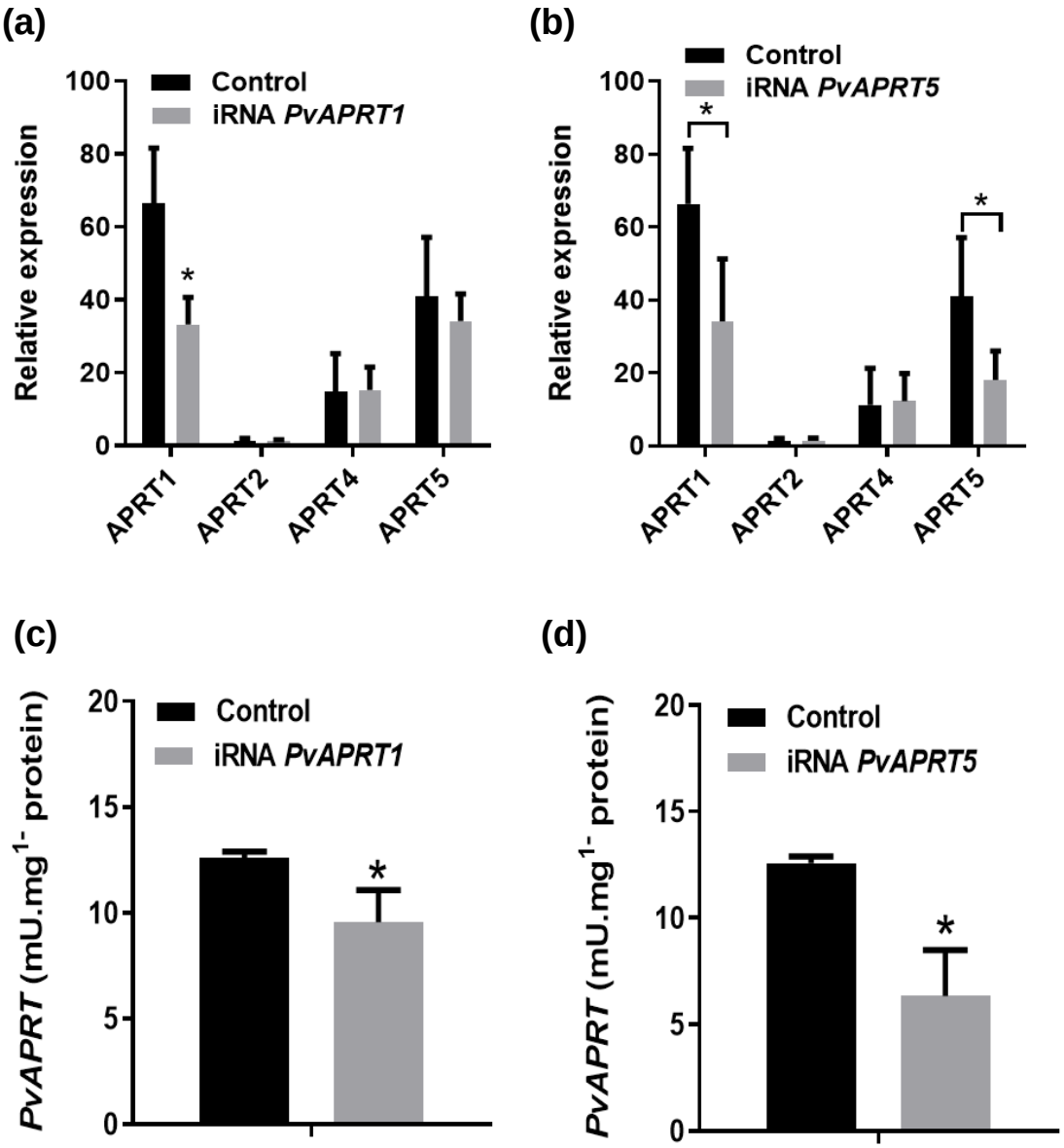
Supplementary Figure S4. Michaelis-Menten and Lineweaver-Burk plots for Km determination of the *P. vulgaris* APRT isoforms. (a) adenine or (b) PRPP plots for APRT1; (c) adenine or (d) PRPP plots for APRT4. (e) adenine or (f) PRPP for APRT5.



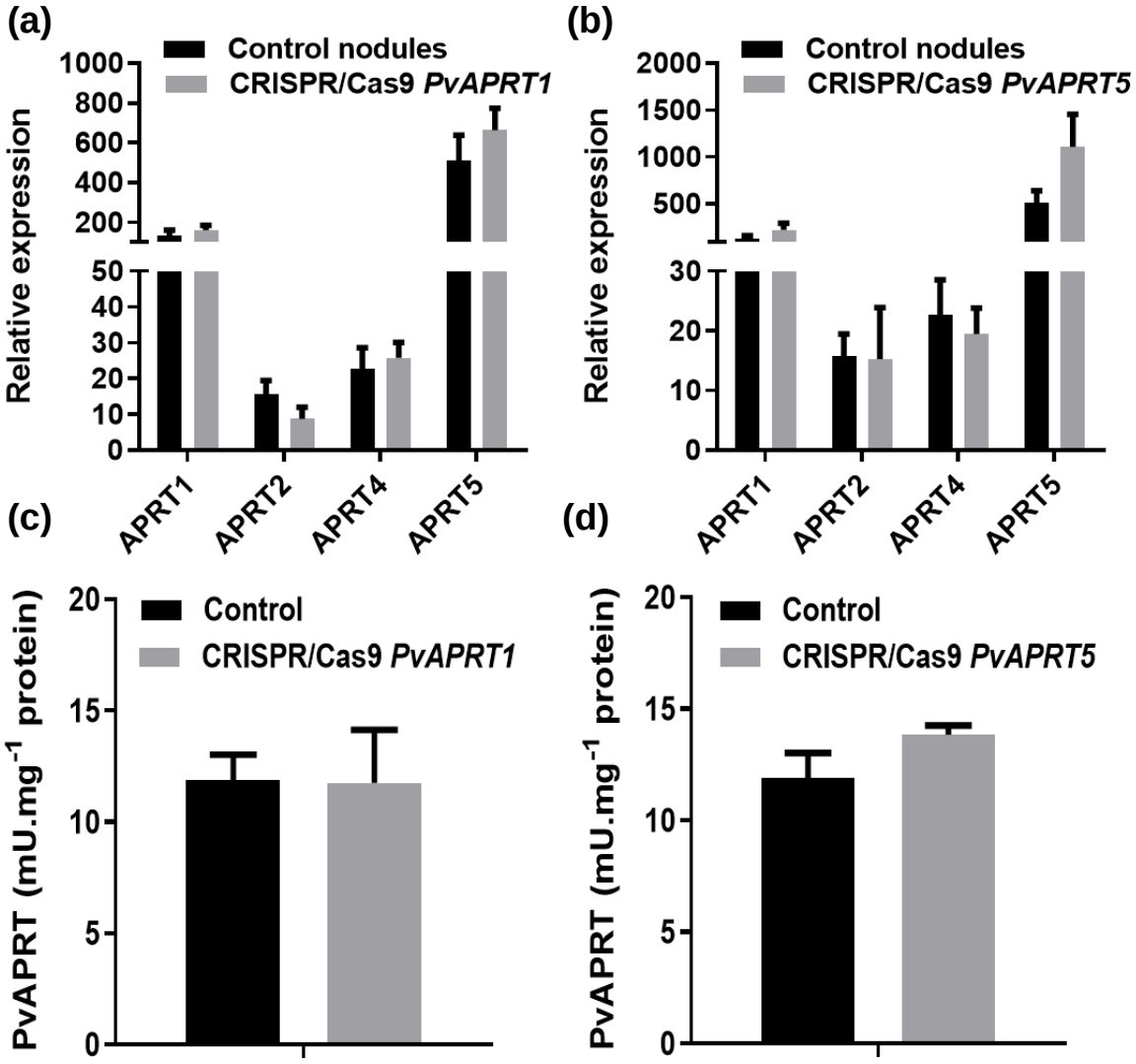
Supplementary Figure S5. Schematic representation of the *cis*-regulatory elements in the promoter sequences of the *PvAPRT1, 2, 4* and *5* genes. The promoter sequences of the *PvAPRT1*, *PvAPRT2*, *PvAPRT4 and PvAPRT5* genes were obtained from Phytozome 13 (https://phytozome-next.jgi.doe.gov/) and the analysis of the motifs performed using the PlanCARE software (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/).



Supplementary Figure S6. Analysis of the relative expression of the *PvAPRT1* and *5* genes in *P. vulgaris* hairy roots. Relative expression of the (a) *PvAPRT1* and (b) *PvAPRT5* gene in CRISPR/Cas9 edited hairy roots.



Supplementary Figure S7. Relative expression of *PvAPRT* genes and APRT activity in iRNA silenced roots. Relative expression of *PvAPRT1*, *PvAPRT2*, *PvAPRT4* and *PvAPRT5* genes in transgenic roots with *PvAPRT1* (a) and *PvAPRT5* (b) genes downregulated by iRNA. Total APRT enzyme activity in transgenic roots with *PvAPRT1* (c) and *PvAPRT5* (d) genes downregulated by iRNA (*$p < 0.05$ and ** $p < 0.005$).



Supplementary Figure S8. Relative expression of *PvAPRT* genes and APRT activity in nodules of CRISPR/Cas9 APRTs targeted roots. Relative expression of *PvAPRT1*, *PvAPRT2*, *PvAPRT4* and *PvAPRT5* genes in nodules of transgenic roots with *PvAPRT1* (a) and *PvAPRT5* (b) genes downregulated by CRISPR/Cas9. Relative APRT activity in nodules of transgenic roots pools with *PvAPRT1* (c) and *PvAPRT5* (d) genes downregulated by CRISPR/Cas9.

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

Primer name	Application	Sequence (5` > 3´)
U6-AscI-F	**CRISPR/Cas9**	CGAGGCGCGCCAGAAATCTCAAAATTCCG
U6-SwaI-F	**CRISPR/Cas9**	CGATTTAAATAGAAATCTCAAAAATTCCG
gRNA-PacI-R	**CRISPR/Cas9**	CGATTAATTAATAATGCCAACTTTGTACA
gRNA-AscI-R	**CRISPR/Cas9**	CGAGGCGCGCCTAATGCCAACTTTGTACA
R1-AP1-1	**CRISPR/Cas9**	GAAGGAGTGGAGCGGCGGCTCAATCACTACTTCGTCTCT
R1-AP1-2	**CRISPR/Cas9**	TGGGATCAAGAAGCAGCGTGCAATCACTACTTCGTCTCT
F2-AP1-1	**CRISPR/Cas9**	GAGCCGCCGCTCCACTCCTTCGTTTTAGAGCTAGAAATAGC
F2-AP1-2	**CRISPR/Cas9**	GCACGCTGCTTCTTGATCCCAGTTTTAGAGCTAGAAATAGC
R1-AP5-712	**CRISPR/Cas9**	GTACCGTACTGTACTCTTCCAATCTACTTGCTCTCT
R1-AP5-1527	**CRISPR/Cas9**	GGCAGCTTCCGTGGTTTGCCAATCACTACTTCGTCTCT
F2-AP5-712	**CRISPR/Cas9**	GGAAGAGTACAGTACGGTACGTTTTAGAGCTAGAAATAGC
F2-AP5-1527	**CRISPR/Cas9**	GGCAAACCACGGAAGCTGCCGTTTTAGAGCTAGAAATAGC
DsAPRT1 Fw	**iRNA**	TGATCCCTGACTTTCCCAAGC
DsAPRT1 Rev	**iRNA**	GTTCCCCCAGTGGCAATAAGA
DsAPRT5 Fw	**iRNA**	GCTCTAGAGGCGCGCCCACTCACAGCCAAAGCT
DsAPRT5 Rev	**iRNA**	CAGGATCCATTTAAATTGCTCTGGAACATTATTCCA
gAPRT1 Fw	**Genomic**	ATGCGGGTTGTGTGTTGCT
gAPRT1 Rev	**Genomic**	CGAGGCGCGCCTAATGCCAACTTTGTACA
gAPRT5 Fw	**Genomic**	TGTCGATCGTTACAGAGACAT
gAPRT5 Rev	**Genomic**	ACACAATCTCTTATTCAACATTC
APRT1-Cla I	**Overexpression**	CCCATCGATATGCGGGTTGTGTGTTGCTCCA
APRT1-Sma I	**Overexpression**	TACCCGGGGGCCGTCACTTTAACTAAGACA
APRT2-Cla I	**Overexpression**	ACCATCGATATGTTTGTCCTAGAAAATGGT
APRT2-Sma I	**Overexpression**	TACCCGGGAAAACCATTCTCTATTTGGCGT
APRT4-Cla I	**Overexpression**	ACCATCGATATGTGTGTATCTTTGCCAAC
APRT4-Sma I	**Overexpression**	TACCCGGGTATCTCCATATATTCCACCAAC
APRT5-Cla I	**Overexpression**	CCCATCGATATGTTTGCAGAAGAGAATGGCCT
APRT5-Sma I	**Overexpression**	TACCCGGGCATGTCTTTATAACATTTATCTGCT
APRT1 Fw	**qRT-PCR**	AACCACGCTGCTTCTTGATC
APRT1 Rev	**qRT-PCR**	GCTTCAACACCTGCAACAAC
APRT2 Fw	**qRT-PCR**	CTCACTTCCCCAAACAAGGA
APRT2 Rev	**qRT-PCR**	ACTTGGCACCAATGCCTAAC
APRT4 Fw	**qRT-PCR**	ATCCTCGTCTTCATGGCATC
APRT4 Rev	**qRT-PCR**	TTCCTGCAACGACAGAAATG
APRT5 Fw	**qRT-PCR**	AGGCAAGGGGATTCTTGTTT
APRT5 Rev	**qRT-PCR**	TGCTGACAGAGTTCCACCTG
ACTINE Fw	**qRT-PCR**	GGAGAAGATTTGGCATCACACGTT
ACTINE Rev	**qRT-PCR**	GTTGGCCTTGGGATTGAGTGGT
UBIQUITIN Fw	**qRT-PCR**	GAGCTCAGACACCATTGACAAC
UBIQUITIN Rev	**qRT-PCR**	CTGGATGTTGTAGTCTGCAAGG

Supplementary Table S2. Metabolite annotation and documentation for GC- and LC-MS data.

Peak no.	Ret. Time	Metabolite name	ES(+) Found m/z	Identification level (A-B)	
M1	1.32	Citric acid	193	A	GC-MS
M2	3.07	Hydroxylamine	133	A	GC-MS
M3	3.10	Glyoxylic acid	160	A	GC-MS
M4	3.34	Glycolic acid	177	A	GC-MS
M5	3.48	Alanine	116	A	GC-MS
M6	3.50	Pyruvic acid	174	A	GC-MS
M7	4.03	Pantethine	220	A	GC-MS
M8	4.26	Guanidine	171	A	GC-MS
M9	4.30	Valine	144	A	GC-MS
M10	4.60	Glycerol	205	A	GC-MS
M11	4.66	Ethanolamine	174	A	GC-MS
M12	4.88	Leucine	158	A	GC-MS
M13	5.13	Isoleucine	158	A	GC-MS
M14	5.24	Glycine	174	A	GC-MS
M15	5.30	Phosphoric acid	299	A	GC-MS
M16	5.45	Proline	142	A	GC-MS
M17	5.47	Urea	189	A	GC-MS
M18	5.55	Glyceric acid	292	A	GC-MS
M19	5.76	Serine	204	A	GC-MS
M20	5.88	Succinic acid	247	A	GC-MS
M21	5.92	Threonine	219	A	GC-MS
M22	5.98	Fumaric acid	245	A	GC-MS
M23	6.10	Maleic acid	170	A	GC-MS
M24	6.24	Nicotinic acid	180	A	GC-MS
M25	6.33	Uracil	241	A	GC-MS
M26	6.36	Beta-alanine	248	A	GC-MS
M27	6.57	Erythrose	205	A	GC-MS
M28	6.60	Homoserine	218	A	GC-MS
M29	6.75	Erythritol	217	A	GC-MS
M30	7.13	Malic acid	233	A	GC-MS
M31	7.32	Gamma-Aminobutyric acid (GABA)	304	A	GC-MS
M32	7.40	Aspartic acid	232	A	GC-MS
M33	7.50	Threonic acid	292	A	GC-MS
M34	7.60	2-isopropylmalate	275	A	GC-MS
M35	7.68	Methionine	176	A	GC-MS
M36	7.98	Glutamine	227	A	GC-MS
M37	8.08	Xylose	307	A	GC-MS
M38	8.23	Glutamic acid	246	A	GC-MS
M39	8.35	Rhamnose	117	A	GC-MS

M40	8.39	Putrescine	174	A	GC-MS
M41	8.46	Fucose	117	A	GC-MS
M42	8.48	2-oxo-glutaric acid	198	A	GC-MS
M43	8.60	Phenylalanine	192	A	GC-MS
M44	8.91	Asparagine	116	A	GC-MS
M45	9.10	Glycerol-2-phosphate	243	A	GC-MS
M46	9.25	Ornithine	142	A	GC-MS
M47	9.30	Glycerol-3-phosphate	357	A	GC-MS
M48	9.35	Glucose	160	A	GC-MS
M49	9.69	Aconitic acid-cis	229	A	GC-MS
M50	9.70	Mannitol	319	A	GC-MS
M51	9.70	Fructose	217	A	GC-MS
M52	9.76	Shikimic acid	204	A	GC-MS
M53	9.80	Mannose	160	A	GC-MS
M54	9.84	Arginine	157	A	GC-MS
M55	9.99	Lysine	156	A	GC-MS
M56	10.00	Isocitric acid	245	A	GC-MS
M57	10.15	Dehydroascorbic acid	316	A	GC-MS
M58	10.26	Galactonic acid	333	A	GC-MS
M59	10.37	Tyramine	174	A	GC-MS
M60	10.62	Myo-inositol	305	A	GC-MS
M61	10.66	D-Gulono-1,4-lactone	333	A	GC-MS
M62	10.72	Allantoin	333	A	GC-MS
M63	10.97	Tyrosine	218	A	GC-MS
M64	11.00	Histidine	154	A	GC-MS
M65	11.60	Adenine	264	A	GC-MS
M66	12.00	Spermidine	174	A	GC-MS
M67	12.70	Fructose-6-phosphate	459	A	GC-MS
M68	12.84	Tryptophan	202	A	GC-MS
M69	13.00	Glucose-6-phosphate	160	A	GC-MS
M70	14	Sucrose	361	A	GC-MS
M71	14.33	alpha-alpha-trehalose	361	A	GC-MS
M72	14.50	Maltose	204	A	GC-MS
M73	15.60	Galactinol	204	A	GC-MS
M74	17.21	Raffinose	207	A	GC-MS
M75	17.60	Adenosine-5-monophosphate	169	A	GC-MS
M76	18.01	cholesterol	129	A	GC-MS
M77	0.61	Dihydroxyacetone phosphate	171	B	LC-MS
M78	0.62	alpha-D-Galacturonic acid 1-phosphate	275	B	LC-MS
M79	0.62	D-Galacturonic acid	275	B	LC-MS
M80	0.63	(S)-alpha-Amino-gamma-butyrolactone hydrochloride	102	B	LC-MS
M81	0.63	Alpha-L-(-)-Fucose-1-Phosphate	245	B	LC-MS
M82	0.63	2-Keto-L-gulonic acid	195	B	LC-MS
M83	0.64	6-phosphogluconic acid	277	B	LC-MS

M84	0.65	Gluconic acid lactone	179	B	LC-MS
M85	0.65	3-Deoxy-D-manno-2-octulosonic acid	239	B	LC-MS
M86	0.66	Conduritol B Epoxide	161	B	LC-MS
M87	0.68	Tartaric acid	149	B	LC-MS
M88	0.69	Uridine 5'-diphosphate	403	B	LC-MS
M89	1.05	Arabinonic acid, gamma-lactone	149	B	LC-MS
M90	1.08	alpha-Ketoglutaric acid	145	B	LC-MS
M91	1.37	Dopamine	152	B	LC-MS
M92	1.49	Pyroglutamic acid	130	B	LC-MS
M93	1.54	NAD	662	B	LC-MS
M94	1.81	Threonyl-proline	217	B	LC-MS
M95	1.85	Citramalic acid	149	B	LC-MS
M96	1.91	Aspartyl-Methionine	263	B	LC-MS
M97	1.99	Methionyl-glutamic acid	279	B	LC-MS
M98	2.13	S-Adenosyl-homocysteine	383	B	LC-MS
M99	2.22	Indole-3-carboxylic acid	116	B	LC-MS
M100	2.71	Inosine	267	B	LC-MS
M101	2.73	Gamma-Glutamyl-S-methylcysteinyl-beta-alanine	334	B	LC-MS
M102	2.99	Bis-gamma-glutamylcysteinyl-bis-beta-alanine	639	B	LC-MS
M103	3.08	Salicylic acid	93	B	LC-MS
M104	3.09	Porphobilinogen	227	B	LC-MS
M105	3.15	Galloyl-glucose	331	B	LC-MS
M106	3.37	Caffeoylglucarate	371	B	LC-MS
M107	3.64	Pantothenic acid	218	B	LC-MS
M108	3.68	Threonyleucine	233	B	LC-MS
M109	3.69	4-Aminobenzoic acid	138	B	LC-MS
M110	3.76	Licoagroside B	431	B	LC-MS
M111	3.84	Zeatin-glucoside	380	B	LC-MS
M112	4.04	Vanilloside	313	B	LC-MS
M113	4.12	Caffeoyl tartaric acid	311	B	LC-MS
M114	4.13	Abscisic acid	153	B	LC-MS
M115	4.18	4-Hydroxymethyl-3-methoxyphenoxyacetic acid	211	B	LC-MS
M116	4.22	Isoleucyl-proline	229	B	LC-MS
M117	4.23	10-Formyl-tetrahydrofolate	472	B	LC-MS
M118	4.28	Feruloylgalactarate	385	B	LC-MS
M119	4.29	5'-Deoxy-5'-Methylthioadenosine	296	B	LC-MS
M120	4.37	Theophylline, anhydrous	181	B	LC-MS
M121	4.38	L-gamma-glutamyl-L-isoleucine	259	B	LC-MS
M122	4.45	Aspartyl-phenylalanine	281	B	LC-MS
M123	4.67	Indole-3-acetic acid	130	B	LC-MS
M124	4.67	Salicylic acid beta-D-glucoside	299	B	LC-MS
M125	4.76	Dihydrophaseic acid glucoside	443	B	LC-MS
M126	4.76	1-O-Feruloyl-glucose	355	B	LC-MS
M127	4.81	p-coumaroyl tartaric acid	295	B	LC-MS

M128	4.87	Karrikin 2	137	B	LC-MS
M129	4.95	Sinapoylgalactarate	385	B	LC-MS
M130	4.95	Kaempferol-glc-glc like	609	B	LC-MS
M131	4.96	Helicin	285	B	LC-MS
M132	4.99	Asparaginyl-Tryptophan	319	B	LC-MS
M133	5.03	3-caffeoyl-Quinic acid	355	B	LC-MS
M134	5.09	3-(2,4-dihydroxyphenyl)-Propanoic acid	183	B	LC-MS
M135	5.31	Fertaric acid	325	B	LC-MS
M136	5.44	p-coumaroyl di-glucoside	487	B	LC-MS
M137	5.58	Jasmonic acid	59	B	LC-MS
M138	5.62	Genistein-glc	431	B	LC-MS
M139	5.80	Riboflavin	399	B	LC-MS
M140	5.82	Phaseolic acid	295	B	LC-MS
M141	5.84	Quercetin-glc-glc-rha	771	B	LC-MS
M142	5.89	Dihydrophaseic acid	281	B	LC-MS
M143	5.91	(-)-11-hydroxy-9,10-dihydrojasmonic acid 11-beta-D-glucoside	389	B	LC-MS
M144	5.97	Phenylpyruvic acid	163	B	LC-MS
M145	5.99	Ampelopsin	321	B	LC-MS
M146	6.07	7-Epi-12-hydroxyjasmonic acid glucoside	387	B	LC-MS
M147	6.17	Quercetin-rha-glc-xyl	741	B	LC-MS
M148	6.26	Daidzein-glc	415	B	LC-MS
M149	6.30	Quercetin 3-O-glucoside 7-O-rhamnoside	611	B	LC-MS
M150	6.42	N6-benzyl-Adenine	226	B	LC-MS
M151	6.44	Quercetin-glc-xyl	595	B	LC-MS
M152	6.53	MeGenistein-glc	445	B	LC-MS
M153	6.56	Cis-3-Hexenyl b-primeveroside	393	B	LC-MS
M154	6.59	p-Coumaric acid	165	B	LC-MS
M155	6.64	O-Hydroxyhippuric acid	196	B	LC-MS
M156	6.67	Kaempferol-rha-glc-xyl	725	B	LC-MS
M157	6.71	Methyl-Quercetin-rha-glc-xyl	755	B	LC-MS
M158	6.71	Phlorizin	435	B	LC-MS
M159	6.74	p-coumaroylmalate	279	B	LC-MS
M160	6.98	Kaempferol-glc-xyl	579	B	LC-MS
M161	7.00	Quercetin-glcA	477	B	LC-MS
M162	7.18	Scopoletin	191	B	LC-MS
M163	7.19	Laricitrin-glc like	493	B	LC-MS
M164	7.20	Quercetin-3-beta-D-glucoside	465	B	LC-MS
M165	7.59	MeDaidzein-glc	429	B	LC-MS
M166	7.62	Kaempferol-glcA	461	B	LC-MS
M167	7.70	Jasmonoyl-L-isoleucine	130	B	LC-MS
M168	7.71	Naringin	581	B	LC-MS
M169	7.74	Quercitrin	449	B	LC-MS
M170	7.74	Trans-Caffeic acid [apiosyl-(1->6)-glucosyl] ester	429	B	LC-MS
M171	7.85	Methyl-kaempferol glucuronide	491	B	LC-MS

M172	8.00	Methylsyringin	385	B	LC-MS
M173	8.00	Trans-3,5-Dimethoxy-4-hydroxycinnamaldehyde	209	B	LC-MS
M174	8.05	Phospho-L-Arginine	255	B	LC-MS
M175	9.47	Azukisaponin VI	1134	B	LC-MS
M176	9.97	Soyasaponin A2	1106	B	LC-MS
M177	10.10	Pseudochelerythrine	333	B	LC-MS
M178	10.37	Soyasaponin A3	958	B	LC-MS
M179	10.38	Licoricesaponin F3	951	B	LC-MS
M180	10.62	Azukisaponin IV	971	B	LC-MS
M181	11.78	Soyasaponin VI - glc	1230	B	LC-MS
M182	12.24	Pisumsaponin I	1028	B	LC-MS
M183	12.33	Olaxoside	939	B	LC-MS
M184	12.43	Soyasaponin II	912	B	LC-MS
M185	12.46	Soyasaponin I	942	B	LC-MS
M186	12.61	Soyasaponin IV	765	B	LC-MS
M187	12.63	Jujubasaponin VI	795	B	LC-MS
M188	12.64	Saponin Oleanolic acid der	926	B	LC-MS
M189	12.81	Putative Soyasaponin bg	909	B	LC-MS
M190	12.90	Putative Soyasaponin beta-A	1038	B	LC-MS
M191	12.93	Soyasaponin VI	1068	B	LC-MS
M192	13.00	Cynarasaponin C	793	B	LC-MS
M193	13.13	Putative Soyasaponin gamma-A	891	B	LC-MS
M194	13.16	Soyasaponin yg	921	B	LC-MS
M195	13.44	Beta-Hydroxylauric acid	215	B	LC-MS
M196	14.66	alpha-Tocopherol acetate	471	B	LC-MS

Ret. Time: Retention time; metabolite name: putative identification of the metabolite; found m/z: mass detected in the experiment; identification level (A; B): (A) standard or NMR and (B) confident match based on MS/MS; Glc: glucose; xyl: xylose; rha: rhamnose.

Supplementary Table S3. Physicochemical properties of APRT proteins from *P. vulgaris* cv. Great Northern "Matterhorn" (PMB-0220).

ID	Protein lenght (aa)	pI	Molecular weight (kDa)	Instability index	Stable or unstable	GRAVY	Aliphatic index	Subcelullar localization
APRT 1	246	8.34	26.96	42.97	Unstable	0.065	103.82	Chloroplast transfer peptide
APRT 1-SP	186	4.93	20.44	26.53	Stable	0.076	112.69	
APRT 2	193	5.29	20.9	19.86	Stable	0.305	114.15	Cellular cytosol
APRT 4	230	7.52	25.37	37.23	Stable	0.39	116.52	Chloroplast transfer peptide
APRT 4-SP	184	6.75	20.36	32.22	Satable	0.113	108.59	
APRT 5	201	6.52	21.84	21.14	Stable	0.155	104.73	Cellular cytosol

(-SP: withouth signal peptide; aa: aminoacid; pI: isoelectric point)

Supplementary Table S4. Metabolomic changes in *P. vulgaris* edited hairy roots

Metabolite group	Metabolite name	Log2Foldchange		*p-value*	
		Control vs CRISPR/Cas9 APRT1	Control vs CRISPR/Cas9 APRT5	Control vs CRISPR/Cas9 APRT1	Control vs CRISPR/Cas9 APRT5
Alcohol	Licoagroside B	-0.33	0.40	0.45	0.34
Aldehyde	Helicin	-0.60	0.92	0.34	0.035*
	Trans-3,5-Dimethoxy-4-hydroxycinnamaldehyde	0.81	1.17	0.08	0.0044**
Alkaloid	Pseudochelerythrine	1.07	1.32	0.11	0.12
	Theophylline, anhydrous	-0.72	0.98	0.38	0.10
Amide	Allantoin	0.60	1.11	0.26	0.23
Amide	Urea	3.96	0.86	0.024*	0.38
	Ethanolamine	0.01	0.02	0.91	0.97
	Putrescine	0.19	0.35	0.80	0.67
	Spermidine	-0.06	0.18	0.93	0.76
	Tyramine	-0.61	0.00	0.43	0.99
	Dopamine	-0.21	0.74	0.76	0.38
Amino Acid	Alanine	1.21	-0.23	0.024*	0.63
	β-alanine	0.48	0.52	0.12	0.32
	Arginine	3.50	4.43	0.21	0.15
	Asparagine	0.63	1.92	0.10	0.13
	Aspartic acid	0.81	0.38	0.22	0.48
	Glutamic acid	1.05	0.83	0.03*	0.21
	Glutamine	0.76	0.52	0.29	0.49
	Glycine	0.38	0.47	0.50	0.48
	Histidine	1.21	0.62	0.11	0.39
	Homoserine	-2.34	-0.75	0.0008**	0.18
	Isoleucine	-0.74	0.44	0.018*	0.64
	Leucine	-0.58	1.33	0.06	0.32
	Lysine	-0.14	-0.04	0.67	0.95
	Methionine	0.23	0.81	0.68	0.33
	Ornithine	0.09	1.56	0.89	0.12
	Phenylalanine	0.03	0.12	0.96	0.84
	Proline	0.26	1.42	0.53	0.14
	Serine	1.60	1.46	0.005*	0.0006**
	Threonine	0.06	0.20	0.92	0.78
	Tryptophan	-2.07	-0.15	0.13	0.89
	Tyrosine	0.36	-0.02	0.58	0.98
	Valine	-0.08	-0.52	0.90	0.44
Azoles	Porphobilinogen	0.17	-1.13	0.84	0.37
	Erythritol	0.30	3.02	0.32	0.15
	Fucose	-0.75	0.38	0.11	0.48
	Galactonic acid	-0.72	0.53	0.17	0.35
	Glyceric acid	0.15	0.18	0.75	0.73
	Glycerol	0.11	0.37	0.82	0.46
	Glycerol-2-phosphate	-2.21	-4.61	0.12	0.11
	Glycerol-3-phosphate	0.88	0.79	0.11	0.11
	D-Gulono-1,4-lactone	-0.13	-0.11	0.63	0.78
	Raffinose	2.06	2.52	0.21	0.014*
	Rhamnose	0.14	0.12	0.74	0.82
	α-α-trehalose	0.93	-0.20	0.10	0.68
	2-Keto-L-gulonic acid	0.51	1.26	0.28	0.003**
	3-Deoxy-D-manno-2-octulosonic acid	0.68	1.23	0.11	0.03*
	5'-Deoxy-5'-Methylthioadenosine	-0.34	-0.51	0.54	0.37
	6-phosphogluconic acid	0.71	0.85	0.21	0.008*

	7-Epi-12-hydroxyjasmonic acid glucoside	1.28	0.01	0.35	0.98
	α-D-Galacturonic acid 1-phosphate	0.54	-0.18	0.50	0.65
	Cis-3-Hexenyl b-primeveroside	1.17	0.09	0.22	0.85
	Conduritol B Epoxide	0.85	1.32	0.07	0.002**
	Dihydrophaseic acid glucoside	-0.07	0.74	0.87	0.08
	Dihydroxyacetone phosphate	0.18	0.56	0.58	0.17
	Feruloylgalactarate	-0.84	0.17	0.42	0.80
	Gluconic acid lactone	-0.01	0.94	0.99	0.10
	Methylsyringin	0.27	0.11	0.41	0.74
	Phlorizin	0.71	1.49	0.34	0.002**
	Pisumsaponin I	-0.10	1.05	0.86	0.08
	Salicylic acid β-D-glucoside	0.42	0.68	0.27	0.20
	Tartaric acid	0.47	0.06	0.35	0.91
	Trans-Caffeic acid [apiosyl-(1->6)-glucosyl] ester	2.54	1.55	0.12	0.04*
	Uridine 5'-diphosphate	-0.82	0.13	0.23	0.74
	Vanilloside	1.62	0.74	0.20	0.14
Carboxylic Acid	2-isopropylmalate	0.60	1.53	0.13	0.07
	Aconitic acid-cis	0.36	0.41	0.77	0.59
	Citric acid	-0.14	-0.36	0.55	0.30
	Fumaric acid	0.57	0.08	0.07	0.76
	γ-Aminobutyric acid (GABA)	0.67	1.32	0.31	0.11
	2-oxo-glutaric acid	2.31	2.64	0.003**	0.04*
	Glycolic acid	0.35	0.74	0.54	0.22
	Glyoxylic acid	-1.28	-3.30	0.09	0.03*
	Isocitric acid	3.61	4.10	0.22	0.001**
	Maleic acid	0.47	-0.13	0.13	0.73
	Malic acid	0.39	0.63	0.24	0.06
	Pyroglutamic acid	1.52	0.95	0.04*	0.41
	Pyruvic acid	2.20	1.69	0.004**	0.06
	Shikimic acid	-0.30	0.72	0.67	0.29
	Succinic acid	0.45	0.28	0.31	0.56
	Threonic acid	1.12	1.53	0.18	0.05
	(-)-11-hydroxy-9,10-dihydrojasmonic acid 11-beta-D-glucoside	0.50	1.38	0.49	0.01*
	(S)-α-Amino-γ-butyrolactone hydrochloride	0.23	0.30	0.67	0.60
	4-Aminobenzoic acid	0.01	-0.94	0.98	0.10
	4-Hydroxymethyl-3-methoxyphenoxyacetic acid	-1.04	-0.24	0.38	0.77
	α-Ketoglutaric acid	0.51	0.68	0.22	0.06
	α-L-(-)-Fucose-1-Phosphate	1.34	0.46	0.01*	0.11
	Citramalic acid	-1.97	-0.02	0.02*	0.96
	O-Hydroxyhippuric acid	-0.21	0.33	0.64	0.37
	p-Coumaric acid	0.43	0.36	0.48	0.55
	Phaseolic acid	0.12	0.20	0.79	0.68
	Phenylpyruvic acid	0.76	1.17	0.17	0.09
	3-(2,4-dihydroxyphenyl)-Propanoic acid	0.10	-0.64	0.79	0.33
	Sinapoylgalactarate	1.69	2.19	0.02*	0.009*
Chlorogenic acid	3-caffeoyl-quinic acid	0.03	-0.33	0.93	0.51

Fatty acid	β-Hydroxylauric acid	0.26	0.27	0.34	0.32
Flavonoid	Ampelopsin	0.99	1.48	0.02*	0.04*
	Arabinonic acid, γ-lactone	-0.45	0.36	0.22	0.26
	Daidzein-glc	0.47	1.71	0.46	0.009*
	Genistein-glc	0.15	1.29	0.69	0.01*
	Kaempferol-glc-glc like	1.28	1.89	0.0002**	0.00002**
	Kaempferol-glc-xyl	0.84	0.15	0.34	0.83
	Kaempferol-rha-glc-xyl	0.32	0.22	0.70	0.83
	MeDaidzein-glc	0.10	0.74	0.80	0.04*
	MeGenistein-glc	-0.06	0.30	0.93	0.60
	Methyl-kaempferol glucuronide	-0.59	-0.73	0.51	0.43
	Methyl-Quercetin-rha-glc-xyl	-3.00	-2.83	0.20	0.21
	Naringin	-0.69	-0.25	0.55	0.76
	Quercetin-3-β-D-glucoside	-1.29	-0.27	0.16	0.73
	Quercetin-glcA	-2.75	1.02	0.11	0.27
	Quercetin-glc-glc-rha	0.64	0.22	0.45	0.85
	Quercetin-glc-xyl	1.28	2.86	0.03*	0.02*
	Quercetin-rha-glc-xyl	0.12	0.45	0.78	0.20
	Quercitrin	-1.02	1.31	0.13	0.04*
Flavonol	Laricitrin-glc like	0.34	0.80	0.36	0.08
Glucoside	p-coumaroyl di-glucoside	-3.65	4.58	0.44	0.00002**
Hormone	Abscisic acid	0.40	1.17	0.50	0.002**
	Indole-3-acetic acid	-0.74	0.28	0.21	0.61
	Indole-3-carboxylic acid	0.01	0.48	0.95	0.06
	Jasmonic acid	-0.55	0.74	0.19	0.05
	Jasmonoyl-L-isoleucine	-0.30	0.71	0.52	0.24
	Karrikin 2	0.20	1.22	0.69	0.05
	N6-benzyl-Adenine	2.31	1.70	0.17	0.03*
	Salicylic acid	0.52	1.19	0.22	0.003**
Hydroxy carboxylic acid	Dihydrophaseic acid	-0.22	1.09	0.55	0.005*
Hydroxyflavone	Kaempferol-glcA	0.03	-0.17	0.97	0.81
	Quercetin 3-O-glucoside 7-O-rhamnoside	-0.10	0.81	0.87	0.13
Lipid	cholesterol	0.39	0.55	0.23	0.20
N Compound	Hydroxylamine	2.48	0.19	0.03*	0.74
	Guanidine	-0.46	-1.59	0.17	0.008*
Nucleoside	Inosine	1.12	1.42	0.14	0.005*
	S-Adenosyl-homocysteine	0.12	1.14	0.83	0.01*
	NAD	-0.40	-0.27	0.43	0.39
Oxoanion	Caffeoylglucarate	-0.06	0.38	0.92	0.46
Peptide	Asparaginyl-Tryptophan	1.40	0.51	0.22	0.32
	Aspartyl-Methionine	-1.36	0.39	0.16	0.51
	Aspartyl-phenylalanine	1.42	0.73	0.04*	0.06
	Bis-γ-glutamylcysteinyl-bis-β-alanine	-0.46	-0.13	0.34	0.82
	γ-Glutamyl-S-methylcysteinyl-β-alanine	0.12	0.31	0.79	0.53
	Isoleucyl-proline	1.06	1.65	0.30	0.02*
	L-γ-glutamyl-L-isoleucine	0.41	-0.07	0.67	0.95
	Methionyl-glutamic acid	-0.20	0.14	0.77	0.79
	p-coumaroyl tartaric acid	-0.82	-0.73	0.29	0.40
	p-coumaroylmalate	-0.56	-0.23	0.55	0.84
	Threonyleucine	0.21	-0.33	0.53	0.40
	Threonyl-proline	0.33	0.28	0.79	0.78
Phenol	Caffeoyl tartaric acid	0.34	1.03	0.52	0.15
	Fertaric acid	-0.55	-0.82	0.60	0.33

Phosphate	Phosphoric acid	1.41	0.21	0.06	0.68
Phosphorus compounds	Phospho-L-Arginine	-0.27	0.30	0.57	0.51
Polymer	Maltose	0.22	1.49	0.72	0.28
	Galloyl-glucose	0.07	0.78	0.90	0.04*
Polyol	Manitol	-0.80	1.19	0.06	0.15
Purine	Adenine	0.56	-0.33	0.04*	0.24
	Zeatin-glucoside	0.72	0.71	0.07	0.14
	Adenosine-5-monophosphate	0.45	2.22	0.28	0.03*
Pyrans	Scopoletin	-0.25	0.15	0.29	0.66
Pyrimidine	Uracil	0.66	0.74	0.10	0.07
Saponin	Putative Soyasaponin βA	0.40	0.75	0.43	0.26
	Putative Soyasaponin βγ	0.12	0.93	0.88	0.22
	Putative Soyasaponin γA	0.89	1.88	0.16	0.01*
	Saponin Oleanolic acid der	0.85	1.40	0.04*	0.01*
	Soyasaponin A2	-1.88	1.02	0.10	0.54
	Soyasaponin A3	0.82	1.13	0.32	0.13
	Soyasaponin I	0.87	1.01	0.15	0.07
	Soyasaponin II	2.77	0.94	0.29	0.15
	Soyasaponin IV	1.30	2.80	0.02*	0.008*
	Soyasaponin VI	0.53	1.46	0.40	0.26
	Soyasaponin VI-glc	2.46	0.45	0.31	0.32
	Soyasaponin γ	1.59	-1.91	0.13	0.03*
Sugar	Erythrose	0.78	2.38	0.04*	0.19
	Fructose	-1.01	0.16	0.26	0.84
	Galactinol	0.43	0.69	0.49	0.27
	Glucose	0.21	1.22	0.68	0.21
	Mannose	0.65	1.66	0.04*	0.18
	Sucrose	0.20	-0.10	0.26	0.77
	Xylose	-0.95	0.80	0.04*	0.37
	α-Tocopherol acetate	0.21	0.11	0.36	0.65
Sugar acid	D-Galacturonic acid	-0.21	-0.46	0.53	0.31
Sugar phosphate	Fructose-6-phosphate	-0.06	0.16	0.90	0.79
	Glucose-6-phosphate	0.28	1.05	0.10	0.001**
Terpenoid	Azukisaponin IV	0.55	2.28	0.33	0.11
	Azukisaponin VI	0.07	0.49	0.88	0.32
	Cynarasaponin C	0.49	2.12	0.42	0.009*
	Jujubasaponin VI	1.97	2.70	0.0003**	0.00034**
	Licoricesaponin F3	0.43	0.69	0.30	0.13
	Olaxoside	1.48	0.92	0.33	0.10
Vitamin	Myo-inositol	-1.08	1.83	0.03*	0.008*
	Nicotinic acid	0.44	0.37	0.14	0.44
	Pantethine	0.73	1.20	0.37	0.20
	10-Formyl-tetrahydrofolate	0.19	0.19	0.46	0.45
	1-O-Feruloyl-glucose	0.47	0.56	0.29	0.27
	Pantothenic acid	1.25	1.42	0.10	0.08
	Riboflavin	0.11	0.60	0.77	0.15
	Dehydroascorbic acid	-0.89	-0.41	0.007*	0.28

Glc: glucose; xyl: xylose; rha: rhamnose. (*$p < 0.05$) and (**$p < 0.005$)

GENERAL DISCUSSION

Common bean *(Phaseolus vulgaris* L), represents an important source of mineral nutrients and proteins in human diets and is considered as an important staple crop worldwide (Broughton *et al.*, 2003; Beebe, 2012). In addition, its ability to establish symbiosis with soil rhizobia to use atmospheric N_2 leads to a lower use of fertilizers and, therefore, environmental, and economic advantages. However, water scarcity is among the factors that most limit agricultural productivity, and it is also the most limiting factor for common bean yields. At present, climate change and its consequent aggravation of droughts episodes are leading to a decrease in yields of the most important crops, including legumes (Ziska *et al.*, 2016; Arora, 2019*b*). However, due to domestication, broad dissemination and breeding practices, a huge degree of genotype adaptations can be found among common bean cultivars (Frahm *et al.*, 2004; Beebe *et al.*, 2013). These better adapted genotypes can be exploited for a more sustainable agriculture under unfavourable conditions. In this work, an in-depth study has been performed to elucidate the molecular, biochemical, and physiological mechanisms used by common bean in response to drought.

The first chapter shows the results published in (López *et al.*, 2020*a*). In this chapter, a transcriptomic analysis was performed on leaves of the landrace *P. vulgaris* PHA-0683, previously characterized as highly resistant to drought (Coleto *et al.*, 2014). The first interesting result was the low number of DEGs observed in the RNA-seq. This was associated with previous results by our group, where it was shown that the PHA-0683 landrace exhibited only mild symptoms of adverse physiological effects under drought conditions (Coleto *et al.*, 2014). Subsequently, the results derived from the transcriptomic analysis revealed that genes associated with the inhibition of the ABA response, such as the genes encoding an (+)-Abscisic acid 8'-Hydroxylase (ABA 8'H) and a phosphatase 2C (PP2C) (Saito *et al.*, 2004; Cutler *et al.*, 2010), had significantly lower levels of expression during drought than under control conditions in this landrace. ABA is the main regulator of osmotic stress responses in plants. Therefore, a decrease in the expression of genes that favours the inhibition of the response mediated by ABA would indicate that this tolerant genotype could maintain an efficient ABA response to face water stress. Moreover, water deficit treatment also induced changes in the expression of genes related to other stress cell signalling pathways. Among them, genes related to the response to phosphate (P) starvation response stood out. To determine if the lack of P in plants,

influenced the tolerance of the PHA-0683 landrace to water stress, a group of plants was supplemented with P one week before the water deficit treatment. Physiological and gene expression analysis revealed that P supplementation was able to abolish the already moderate detrimental effects of drought in *P. vulgaris* PHA-0683 plants.

Domestication, global expansion, and local agricultural practices have led to the development of a wide variety of common bean genotypes with varying degrees of resistance to water stress (Frahm *et al.*, 2004; Beebe *et al.*, 2013). In the second chapter the molecular and physiological responses to drought in common bean landrace PHA-0683, with high tolerance to drought used in chapter 1, and PMB-0220 Great Northern "Mattherhorn" common bean, which is a market class breeding line, were compared and the results were published in (López *et al.*, 2020*b*).

Results of the physiological analysis of the drought effects in the two genotypes cultivated in symbiosis with N_2-fixing rhizobia showed that, as previously published in chapter 1, the landrace PHA-0683 did not show differences between control plants and plants subjected to drought in their leaf water content (RWC). Nevertheless, there was a slight reduction in the biomass of the aerial part, which could be correlated with the slight reduction in the number of fruits after 10 days of drought (Riveiro, 2012). On the contrary, common bean PMB-0220 breeding line showed a significant decrease in shoot biomass and a slight increase in root biomass under drought conditions compared to control conditions. Among the physiological symptoms of drought stress is leaf senescence, marked by a decrease in chlorophyll content. Determination of chlorophyll concentration in the two common bean genotypes showed that chlorophyll degradation occurred earlier in the PMB-0220 than in the PHA-0683 plants submitted to water stress. These differences between two common bean genotypes confirmed that PMB-0220 was less tolerant to water stress than PHA-0683, as was previously reported in (Riveiro, 2012).

The different degree of tolerance to drought between the two genotypes of *P. vulgaris* could indicate differences in the expression of key genes in the response to water stress. The analysis of the expression levels of genes related to ABA response, showed both quantitative and qualitative differences between the two genotypes. Specifically, in PHA-0683 landrace the expression levels of genes involved in inhibition of the ABA-mediated response and ABA-degradation, respectively, as type 2C protein phosphatases and ABA hydroxylase (ABA 8'H) decreased under drought conditions. In contrast, in the PMB-0220 line the expression of a gene encoding a 9-cis-epoxycarotenoid dioxygenase

(NCED3), which is a key factor in ABA synthesis (Vishwakarma *et al.*, 2017), was promoted by drought. Also, the expression of genes coding for MYC and MYB transcription factors involved in the responses to abiotic stress was different among the two common bean genotypes. Among the transcription factors that showed qualitative differences in their expression pattern were *PvWRKY53* and *PvWRKY57*, associated with induction of senescence (Abe *et al.*, 1997; Miao *et al.*, 2004). These genes had higher expression levels under drought conditions in common bean PMB-0220, which coincides with the decrease in the chlorophyll content under conditions of water stress. In contrast, there were no differences in the expression levels of these two genes in PHA-0683 landrace. All these results indicate that drought tolerance is regulated by different molecular mechanisms between the two genotypes (López *et al*., 2020b).

In subsequent experiments in chapter 3 (López *et al.*, 2023), we focused our analysis on the responses of the common bean commercial line Great Northern "Mattherhorn" PMB-0220, with intermediate tolerance, used in chapter 2, to search for strategies to increase the drought tolerance and nitrogen fixation in this marked-class and highly productive breeding line. Common bean belong to the group of ureidic legumes, which use fixed nitrogen in nodules for the *de novo* synthesis of purine nucleotides, subsequently oxidized to produce the ureides, allantoin and allantoate, which are the main nitrogen transport and storage molecules in these crops (Atkins, 1991; Zrenner *et al.*, 2006; Werner and Witte, 201; Díaz-Leal *et al.*, 2012). Recently, there have been numerous studies showing that ureides also help in the protection of plants, and that induction of ureide formation could be an important advantage under adverse conditions (Brychkova *et al.*, 2008; Alamillo *et al.*, 2010; Irani and Todd, 2016). However, nowadays many farmers prefer the use of inorganic nitrogen fertilizers that ensure a good harvest, despite the advantages of N_2-fixation (Araújo *et al.*, 2015). This is because the symbiosis with rhizobia is particularly sensitive to different adverse conditions such as drought (King and Purcell, 2005). Our hypothesis was that symbiotic nitrogen fixation and the consequent accumulation of ureides should help to ameliorate the adverse effects of drought in common bean.

In chapter 3, the response to drought in common bean plants grown under N_2-fixation or fertilized with 10mM NO_3^- was studied. Currently, the combination of metabolomics and transcriptomics allows to obtain a better understanding how different genotypes act against different types of stress (Scossa *et al.*, 2021). Therefore, in this chapter 3, transcriptomic and metabolomic changes in the PMB-0220 common bean plants

subjected to 10 days of drought and cultivated under symbiosis or fertilized with nitrate were compared. Furthermore, the results obtained by the -omics technologies were supported with a physiological study carried out after 10 days of drought and throughout a period of rehydration, to determine the degree of recovery from drought allowed by each type of crop and growth (López *et al.,* 2023).

The first interesting result from the differential expression of genes in response to drought was that the PMB-0220 plants cultivated under nitrate fertilization have as many as twice gene expression changes than those of plants growing under N_2-fixation. However, plants under N_2-fixation although exhibited less DEGs, they had a higher expression of genes related to ABA-synthesis than NO_3^- fertilized ones, thus agreeing with the results in chapter 2. Even when comparing control conditions, N_2-fixing plants had significantly higher expression of ABA synthesis genes in the absence of stress, compared to nitrate-fertilized plants. In fact, the subsequent metabolomic analysis showed a significant accumulation of ABA in N_2-fixing plants subjected to drought. Accumulation of ABA was much lower in nitrate-fertilized plants subjected to drought. In addition to ABA, the metabolomic analysis also showed the accumulation of other drought defence metabolites. Among them, there was accumulation of the ureide allantoin and soluble sugars, and other metabolites that maintain the osmotic balance in the plant, such as raffinose and proline. On the contrary, plants fertilized with NO_3^- had a considerably lower accumulation of these metabolites during the drought.

A higher ABA-mediated response promotes plant senescence. In chapter 2 it was published (López *et al.*, 2020*b*) that water stress promoted leaf senescence in N_2-fixing plants of PMB-0220 line. In addition, the RNA-seq done in chapter 3 (López *et al.,* 2023), showed that N_2-fixing plants significantly induced the expression of coding STAY-GREEN1 coding gene, among others, responsible for chlorophyll degradation (Luo *et al.*, 2013). In contrast, the plants fertilized with NO_3^- did not show a degradation of their chlorophylls, or the expression of genes that induce senescence as observed in the N_2-fixing plants characterized in chapter 2 and 3. In addition, in chapter 3 we show that N_2-fixing plants accumulate more allantoin under drought conditions than the nitrate-fertilized plants. Ureides are also synthesized in these and other plants under stress conditions that involve the mobilization of nutrients, as a form of utilization and recycling of organic carbon and nitrogen (Díaz-Leal *et al.*, 2012). These results indicate that under water stress, N_2-fixing plants promote a progressive senescence in their vegetative tissues,

favouring the transport and recycling of metabolites towards younger tissues, such as developing leaves, flowers, and fruits. In contrast, leaves from NO_3^--fertilized plants support the stress without apparent activation of nutrient recycling to the youngest tissues. Subsequently, physiological analysis and recovery experiments revealed that N_2-fixing plants recovered their photosynthetic capacity and maintained seed production through rehydration after drought, while fertilized plants did not. This confirms that the enhanced remobilization response to developing organs observed in N_2-fixing plants allowed these plants to overcome drought stress symptoms better than NO_3^- fertilized plants (López *et al.*, 2023).

As previously shown chapter 3, ureides from the degradation of purine nucleotides and, accumulated to higher levels in N_2-fixing plants, may play a key role in drought tolerance, either by better remobilization of nutrients to the youngest tissues or by favouring the ABA synthesis in plants. The concentration of purine nucleotides, from which ureides are generated, is regulated by the *de novo* synthesis pathway and the purine salvage pathways. The *de novo* synthesis route of nucleotides in common bean has already been characterized in our work group (Coleto *et al.*, 2016). However, the study of the two key enzymes in the recycling pathways, adenine phosphoribosyltransferase (APRT) and hypoxanthine guanine phosphoribosyltransferase (HGPRT) in plants has been very scarce to date. Transcriptomic analysis of chapter 3 (López *et al.*, 2023), showed a significant induction under water stress conditions of the genes encoding the APRT protein, which is responsible for adenine salvage to the AMP purine nucleotide (Ashihara *et al.*, 2018). Therefore, in chapter 4, we performed the molecular and biochemical characterization of the APRT protein included in objective 3 listed in the introduction of this thesis work. The characterization of the second enzyme HGPRT has been also approached, but since it has not been finished yet, it is not included in this Thesis work.

In chapter 4, we show the characterization the four adenine phosphoribosyltransferase (APRT) proteins found in common bean, whilst a more profound functional analysis has been performed for the two proteins, PvAPRT 1 and PvAPRT5 that, surprisingly, showed the highest expression and activity levels in nodule tissues, where mainly the *de novo* synthesis of purine nucleotides takes places (Coleto *et al.*, 2016). Activity of APRT catalyzes the salvage of adenine nucleobase to the nucleotide adenosine monophosphate (AMP) and, early studies in plant APRTs have shown that the same enzyme can act in the conversion of active cytokinin nucleobases to inactive cytokinin nucleotides (Moffatt

et al., 1991; Allen et al., 2002; Zhang et al., 2013). Using CRISPR/Cas9 targeted mutation in the respective *PvAPRT1* and *PvAPRT5* genes and metabolomic analysis of the mutant hairy roots, we found that, although PvAPRT1 and 5 have redundant functions catalyzing the conversion of adenine to AMP and transformation of active cytokinin to inactive cytokinin ribotides, these two isoforms also show a certain degree of specialization, with a greater participation of APRT1 in the recovery of adenine in plastids, while APRT5 has a greater role in the regulation of cytokinin activity in the cytosol. Metabolite analysis in the mutant roots also indicated that APRTs have a prominent role in abiotic and biotic stress responses, since their alteration induces the accumulation of stress related phytohormones and secondary metabolites. Moreover, reduction of these two APRTs lead to altered root growth as well as nodule biomass, thus indicating that APRTs functions are required for the proper development of roots and nodules, and in the response to stress.

In summary, this Thesis shows that common bean genotypes of contrasting drought resistance deploy different strategies to tolerate drought stress, that efficient ABA responses are key in the tolerance, and that symbiosis with nitrogen fixing rhizobia highly improves the drought response in a highly productive market-class common bean line. Finally, adenine salvage enzymes have been shown to play a relevant role in regulating root growth, nodulation, and stress responses.

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CONCLUSSIONS

1. The analysis of the molecular mechanisms of drought tolerance demonstrated that keeping activated ABA response and inducing phosphate remobilization are key factors determining the drought resistance of *P. vulgaris* landrace PHA-0683.

2. Expression patterns in response to drought of key regulatory genes *P. vulgaris* drought-resistant PHA-683 landrace and market class Great Northern PMB-0220 breeding line, indicated that the different molecular responses to drought might explain the different degree of tolerance of the two *P. vulgaris* genotypes.

3. Common bean PMB-0220 growth under N_2-fixation and with higher accumulation of ureides promotes a more effective molecular and physiological response to drought conditions than NO_3^--fertilized plants and lower accumulation of ureides.

4. Symbiotic N_2-fixation condition favours that *P. vulgaris* PMB-0220 breeding line plants can be recover from drought stress by subsequent rehydration. Thus guarantees a good harvest and contributes to a more ecological and sustainable agriculture.

5. Gene editing using CRISPR/Cas9 and metabolomic analysis of the targeted roots indicates that although PvAPRT1 and 5 have redundant functions and catalyse the conversion of adenine to AMP and the inactivation of cytokinins, the two isoforms also show some degree of specialization, with APRT1 catalyzing the recycling of adenine in plastids, while APRT5 acts mainly in the regulation of cytokinin activity in the cytosol.

6. Application of transcriptomic and metabolomic analysis coupled to gene editing allows the identification of key regulatory factors and the functional analysis of gene families than can be used in future *P. vulgaris* crop improvements.