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TESIS DOCTORAL

Common bean T2 S-like ribonucleases during situations of high nutrient mobilization

Ribonucleasas T2 S-like de judía en situaciones de alta movilización de nutrientes

Memoria presentada para optar al grado de Doctor por la Universidad de Córdoba por:

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DOCTORANDA/O

Mª Mercedes Díaz Baena

TÍTULO DE LA TESIS

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La presente Tesis Doctoral se ha realizado dentro del Programa de Doctorado de "Biociencias y Ciencias Agroalimentarias" y dentro del grupo BIO-115 del Área de Fisiología Vegetal de la Universidad de Córdoba. La Tesis cumple con los requisitos de la legislación vigente en cuanto a normativa del Programa de Doctorado, su relación con el mismo, así como en la originalidad del trabajo realizado. La doctoranda Mª Mercedes Díaz Baena ha realizado su actividad investigadora con un alto grado de implicación e interés, cumpliendo tanto con el Plan de Investigación como el de Formación que exige la normativa.

En su Tesis Doctoral, la doctoranda ha utilizado una amplia gama de técnicas tanto moleculares como bioquímicas con las que no solo ha adquirido conocimientos técnicos y teóricos para abordar con éxito los objetivos de esta Tesis, sino que ha obtenido una formación sólida que garantiza su solvencia e independencia científica. Es destacable que durante el desarrollo de la Tesis se ha debido de superar las limitaciones impuestas por la situación de pantemia vivida, lo que ha ralentizado en parte la consecución de los objetivos planteados.

En esta Tesis se ha analizado la implicación de 4 genes identificados como ribonucleasas T2 S-like en judía, una leguminosa con altas implicaciones tanto agronómicas como económicas, en distintas situaciones de movilización de nutrientes bajo la hipótesis de que los ácidos nucleicos pueden jugar un papel importante suministranto nutrientes en estas situaciones de alta movilidad.

La investigación llevada a cabo por la doctoranda ha dado lugar a 3 artículos, 2 de ellos publicados en revistas incluidas en "Science Citation Index" bajo la modalidad de "open acces" y situadas en el primer cuartil de su área, y el tercero que se encuentra en segunda ronda de revisión y que se indican a continuación:

Diaz-Baena et al. (2021). Agronomy 11(3), 490; https://doi.org/10.3390/agronomy11030490
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Además, los resultados obtenidos durante el desarrollo de esta Tesis han abierto nuevos frentes de investigación dentro del grupo y que se están desarrollando en la actualidad.

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

Córdoba, a 20 de marzo de 2024

Las/los directoras/es

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Nombre y Apellidos

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A Ari.

TABLE OF CONTENTS

RESUMEN

Los ácidos nucleicos son compuestos orgánicos relativamente abundantes y con funciones esenciales dentro de los seres vivos. Además, debido a su contenido en carbono, nitrógeno y fósforo, pueden desarrollar un papel importante como reservorio de nutrientes y jugar un papel fundamental en situaciones de alta movilización de nutrientes. Entre los ácidos nucleicos, el ARN es la molécula más abundante en las células vegetales, representando alrededor del 85% del total de ácidos nucleicos y el 47% del total de fósforo.

Las ribonucleasas son las enzimas que catalizan la degradación de ARN en compuestos más pequeños; siendo la familia T2 la más extendida entre los seres vivos. En plantas, las ribonucleasas T2 se clasifican en 2 subfamilias (S y S-like) y 3 clases e intervienen en diversos procesos tal y como defensa, reciclaje de nutrientes, reconocimiento o rechazo del polen o mantenimiento de la homeostasis. En este trabajo se ha estudiado en judía la implicación de las ribonucleasas de clase I (representadas en judía por los genes *PvRNS1, PvRNS2 y PvRNS3) y de clase II (PvRNS4)* que componen la familia S-Like.

El objetivo principal de este trabajo ha sido determinar el papel de los ácidos nucleicos como precursores de biomoléculas en situaciones de alta movilización de nutrientes. Para ello las ribonucleasas T2 S-like se han analizado durante la ontogenia de los cotiledones, desde el primer día tras el inicio de la imbibición hasta el día 10, coincidiendo con la senescencia de estos. Los genes *PvRNS1*, *PvRNS2* y *PvRNS4* incrementaron su expresión en la fase de movilización de nutrientes en cotiledones, lo que coincidió con un aumento en la actividad ribonucleasa en esta fase. Estos resultados establecen que las ribonucleasas T2 S-like están involucradas en el reciclaje de ARN en los cotiledones durante el desarrollo de las plántulas.

El metabolismo de ácidos nucleicos también se ha determinado en otro proceso que implica alta movilización de nutrientes, el desarrollo del fruto. La actividad

y expresión de nucleasas se detectó principalmente en la cubierta de las semillas, mientras que, en el caso de las ribonucleasas, además de en la cubierta de las semillas, también se detectó en valvas. En el caso de las nucleotidasas y las nucleosidasas, se determinó una mayor actividad y expresión en valvas y en la cubierta de las semillas. En conjunto, los datos obtenidos permiten concluir que existe un intenso metabolismo de nucleótidos durante la fase de llenado de semillas, con una especial implicación de las cubiertas de las semillas en el proceso.

La actividad ribonucleasa fue igualmente elevada en la testa de la semilla durante la germinación de la semilla. Esta elevada actividad se correlacionó con un alto nivel de expresión de la ribonucleasa *PvRNS3*.

La aparente implicación de *PvRNS3* en la formación de la semilla en frutos, así como en la posterior germinación de la semilla, condujeron a la sobreexpresión de la proteína codificada por este gen como proteína recombinante en *E.coli*. La proteína purificada presentó actividad degradadora de ARN total, ARNr ARNt, pero no degradó ADN de simple o doble cadena, lo que confirma que se trata de una ribonucleasa funcional.

ABSTRACT

Nucleic acids are relatively abundant organic compounds with essential functions within living organisms. In addition, due to their carbon, nitrogen and phosphorus content, they can play an important role as a reservoir of nutrients and play a key role in situations of high nutrient mobilization. Among nucleic acids, RNA is the most abundant molecule in plant cells, representing about 85% of total nucleic acids and 47% of total phosphorus.

Ribonucleases are the enzymes that catalyse the degradation of RNA into smaller compounds; being the T2 family the most widespread among living organisms. In plants, T2 ribonucleases are classified into 2 subfamilies (S and S-like) and 3 classes and are involved in several processes such as defence, nutrient recycling, pollen recognition and maintenance of homeostasis. In this work, the involvement of class I (represented in bean by the *PvRNS1*, *PvRNS2* and *PvRNS3* genes) and classs II (*PvRNS4*) ribonucleases that compose the S-Like family has been studied in common bean.

The main objective of this work was to determine the role of nucleic acids as biomolecules precursors in situations of high nutrient mobilization. For this purpose, T2 S-like ribonucleases were analysed during cotyledon ontogeny, from the first day after the start of imbibition until day 10, coinciding with cotyledon senescence. *PvRNS1*, *PvRNS2* and *PvRNS4* genes increased their expression in the nutrient mobilization phase in cotyledons, coinciding with an increase in ribonuclease activity during this phase. These results establish that T2 S-like ribonucleases are involved in RNA turnover in cotyledons during seedling development.

Nucleic acid metabolism has also been determined in another process involving high nutrient mobilisation, fruit development. The activity and expression of nucleases was detected mainly in the seed coat, while in the case of

ribonucleases, in addition to the seed coat, it was also detected in valves. In the case of nucleotidases and nucleosidases, higher activity and expression was found in valves and seed coat. Overall, the data obtained lead to the conclusion that there is an intense nucleotide metabolism during the seed filling phase, with a special involvement of the seed coat in the process.

Ribonuclease activity was also elevated in the testa during seed germination. This high activity correlated with a high level of *PvRNS3* ribonuclease gene expression.

The apparent involvement of *PvRNS3* in seed formation in fruits, as well as in subsequent seed germination, led to overexpression of the protein encoded by this gene as a recombinant protein in *E.coli*. The purified protein exhibited total RNA, rRNA and tRNA degrading activity, but did not degrade single or double-stranded DNA, confirming that it is a functional ribonuclease.

1. INTRODUCTION

1. Common bean

Common bean (*Phaseolus vulgaris L.*) is a legume that belongs to the genus *Phaseolus*, with the following taxonomic classification: Class Dicotyledoneae, Subclass Rosidae, Superorder Fabanae, Order Fabales, Family Fabaceae, and Subfamily Papilionoidae. It is a self-pollinating plant with a relatively small genome of 650 Mbp, diploid, and organised in 11 chromosomes (De Ron et al., 2015).

The history of cultivated beans can be traced back to their origins in two distinct centres of diversity. Primarily, the Mesoamerican gene pool developed within the core of Central America. Secondly, the Andean gene pool originated in the mountainous regions of the Andes in South America. Each of these gene pools is the outcome of a wholly independent domestication process, indicating that they evolved separately over millennia (Razvi et al., 2017). The divergence in domestication history has given rise to a remarkable diversity, encompassing a wide range of morphological traits and genetic features within common bean. This diversity is particularly evident in growth habits, seed characteristics (including size, shape, and colour), maturation periods, and the presence of alleles conferring resistance to both biotic and abiotic stress factors. This variability enables its cultivation in a wide range of farming systems and diverse environments, spanning across regions such as America, Africa, the Middle East, China, and Europe (Nadeem et al., 2021).

Ensuring food security stands out as one of the paramount challenges we will face in the coming decades. According to the FAO's 2018 assessment of global population growth, they anticipate a projected increase of over 2 billion individuals by the year 2050 (FAO, 2018). As a result, the demand for food will increase. In this context, legumes are often seen as key commodities for improving food security since they offer a relatively cost-effective source of amino acids and other essential nutrients, such as minerals, compared to livestock and dairy products (Jensen et al., 2012). Common bean is the third most significant legume crop used in food worldwide, surpassed only by soybeans (*Glycine max* L.) and peanuts (*Arachis hypogea* L.) (De Ron et a., 2015), and the

first if we consider only direct consumption by humans (Semba et al., 2021). In developing countries, it constitutes a substantial portion of the protein intake, serving as staple foods with a per capita consumption that can reach 40 kg per year (Hayat et al., 2014).

The significance of this crop lies in the fact that its seeds have a high protein content, along with micronutrients and vitamins that offer significant health benefits to consumers, making it a crucial source of food (Karavidas et al., 2022). They can play a crucial role in alleviating malnutrition and combined with cereals can be a substitute for animal protein in diets (Mecha et al., 2018). Common bean has gained significant attention as a functional food in recent times, owing to its potential health benefits and its role in the prevention of human diseases. Notably, its incorporation into the diet has been linked to a decreased risk of conditions such as obesity, diabetes, cardiovascular diseases, and colon, prostate, and breast cancers. These health advantages can be attributed to its substantial fibre and starch content, its capacity to regulate blood glucose levels and gastrointestinal function, as well as its antioxidant properties derived from the presence of phenolic compounds and proteins (De Ron et al., 2015).

Based on the composition of the nitrogen compounds they transport from nodules to the aerial parts of the plant, leguminous plants are classified into two categories, ureidic and amidic, (Atkins, 1991). Common bean is categorized as a ureidic legume because it transports nitrogen fixed in nodules to the aerial parts as ureides. Ureides (allantoin and allantoate), which come from the oxidation of purines, are suitable molecules for the transport and storage of nitrogen (Roy et al., 2020) as they are relatively soluble, stable, and less energetically costly than other nitrogen transporting molecules (Smith and Atkins, 2002). In conditions involving nitrogen fixation, ureides can constitute more than 90 % of the nitrogen content in the xylem of common bean plants (Diaz-Leal et al., 2012). Furthermore, legumes cultivation offers both economic and ecological advantages, as it can acquire a significant portion of the nitrogen needed for its growth by establishing symbiotic relationships with nitrogen-fixing bacteria of the genus *Rhizobium* (Todd et al., 2006; Andrews and Andrews, 2017). This

nitrogen-fixing capability not only enriches the soil but also reduces the requirement for chemical fertilizers, thereby contributing to environmental preservation and the promotion of sustainable agricultural practices (Karavidas et al., 2022). Besides, ureides exhibit considerable potential as nitrogen transport molecules due to their elevated nitrogen-to-carbon (N/C) ratio; with a N/C ratio of 1, these compounds conserve photosynthate much more effectively than amides, which have N/C ratios around 0.5 (Todd et al., 2006). Ureides have been involved in processes such as seedling development (Quiles et al., 2009; 2019) or leaves senescence (Lambert et al., 2017). Furthermore, a role for ureides have been described in stress situations (Irani and Todd, 2016; Baral and Izaguirre-Mayoral, 2017; Kaur et al., 2021).

2. Nucleic acids catabolism

The degradation of nucleic acids, a highly regulated biological process involving the fragmentation of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) molecules, is presumed to play a fundamental role in the redistribution and recycling of essential nutrients within biological systems (Ding et al., 2021). This process is essential for releasing critical biochemical components, such as nucleotides and nitrogenous bases, which can be reused for the synthesis of new nucleic acid molecules or to produce essential metabolites. Additionally, nucleic acid degradation can also release carbon, nitrogen, and phosphorus, crucial elements for the growth and development of organisms. Carbon is essential for plants, as it plays a fundamental role in photosynthesis, where it is used to form carbohydrates and store energy. It also constitutes key cellular components, regulates growth, and plays a role in gas exchange through the leaf stomata (Martínez et al., 2008). Nitrogen is an essential component in plants as it is a part of numerous biomolecules such as amino acids, nucleotides, chlorophyll, vitamins, coenzymes, and hormones (The et al., 2021). Phosphorus is another indispensable component in plant development. It also plays a role in energy storage and transfer (as it is part of energy molecules like ATP), in the synthesis of sugars, phospholipids, and nucleic acids, and is a component of

macromolecules such as nucleic acids and phospholipids. Therefore, phosphorus' function extends to all physiological processes (Fernández, 2007; Malhotra et al., 2018). However, despite extensive research, the physiological role of nucleic acids in the recycling of essential nutrients still holds uncertainties and has not been completely clarified to date.

In situations of high nutrient demand, nucleic acids could provide the essential elements to maintain vital processes and ensure the plant's survival. Two processes with high demand for nutrients are fruit formation and development, and germination and post-germinative growth. These two processes have been analysed in this doctoral thesis.

During the formation of the reproductive tissue, a significant amount of nutrients is demanded from the adult part (Martin and Rose, 2014). Fruit development can be divided into three phases: an early phase, where plant structures begin to develop and the maximum number of cells is reached. A seed-filling phase, where cells expand, the seeds enlarge, and dry matter begins to accumulate (Gehring et al., 2004; Ali et al., 2022); and a maturation phase where the fruit dries, seeds start to dehydrate, and dormancy begins (Ali et al., 2022). The formed seed will consist of three tissues: seed coat, embryo, and endosperm. In dicotyledons, the endosperm is usually ephemeral, with the embryo occupying most of the seed (Chaudhury et al., 2001). Besides acting as a protective barrier for the embryo, during this phase, the seed coat stores hundreds of proteins, nutrients, and other molecules that remain intact throughout the dormancy process, ensuring the embryo's survival during germination and success in its growth (Godwin et al., 2017; Grafi, 2020).

Germination of seeds also requires high nutrient mobilization. Germination starts with the absorption of water by the seed, a process called imbibition, and ends with the emergence of the radicle (Rajjou et al., 2012) In this stage, the seed reactivates its vital functions, and the mobilization of stored nutrients becomes crucial (Rosental et al., 2014). Cotyledons are the main reserve organs, which provides the nutrients necessary for the development of this process. During germination, the seed coat needs to weaken, allowing it to open, and could

provide components that contribute to resistance against biotic and abiotic stress factors (Ndakidemi and Dakora, 2003), in addition to supplying nutrients that enter the seed during imbibition (Raviv et al., 2017). Throughout post-germinative development, the nutrients stored in the cotyledons are transported to the seedling, where they are used for respiration, cell division, and elongation, leading to a young plant that no longer depends on seed reserves (Nonogaki et al., 2010).

As indicated before, the degradation of nucleic acids involves the action of several enzymes and the release of compounds that can serve as a source of nutrients during high demand situations. This process is crucial for the regulation of cellular metabolism and to obtain energy at critical moments. The main intermediates in the process of nucleic acids degradation are indicated in the Figure 1.

Purine nucleotide catabolism initiates with the dephosphorylation of GMP by an unidentified GMP phosphatase (Heineman et al., 2021). The product of this reaction is guanosine, which is then metabolized into xanthosine (Heineman et al., 2021; Witte and Herde, 2020). Subsequently, xanthosine undergoes hydrolysis through nucleoside hydrolases resulting in the formation of xanthine and ribose (Baccolini and Witte, 2019; Delgado-Garcia et al., 2021). Xanthine is then oxidized by xanthine dehydrogenase into urate, which subsequently undergoes degradation through a series of enzymatic steps that release nitrogen in the form of ammonia from the nucleobase and leading to the production of glyoxylate (Baccolini and Witte., 2019). Notably, a recently discovered highly specific cytosolic enzyme, xanthosine monophosphate phosphatase (XMPP), exclusively participates in the dephosphorylation of xanthosine monophosphate to xanthine (Heineman et al., 2021). This enzyme is exclusive to plant nucleotide catabolism. The distinctions between plants and other eukaryotes are as follows: In plants, the synthesis of purine nucleotides, specifically AMP, occurs within chloroplasts, whereas in mammals and yeasts, it takes place in the cytosol. However, the conversion of AMP to GMP in plants occurs in the cytosol.

Notably, plants possess the ability to completely degrade purine nucleotides, breaking down the purine ring (Heineman et al., 2021).

Pyrimidine nucleotide catabolism starts with the dephosphorylation of UMP and cytidine monophosphate (CMP) by currently unidentified phosphatases, resulting in the corresponding ribonucleosides, uridine and cytidine, respectively. Subsequently, cytidine undergoes deamination through the action of a cytidine deaminase to give rise to uridine (Chandel, 2021). Uridine can be enzymatically hydrolysed to yield uracil and ribose (Baccolini and Witte, 2019). The uracil, in turn, can be degraded into β-aminoisobutyrate, which may participate in various other metabolic reactions (Witte and Herde, 2020).

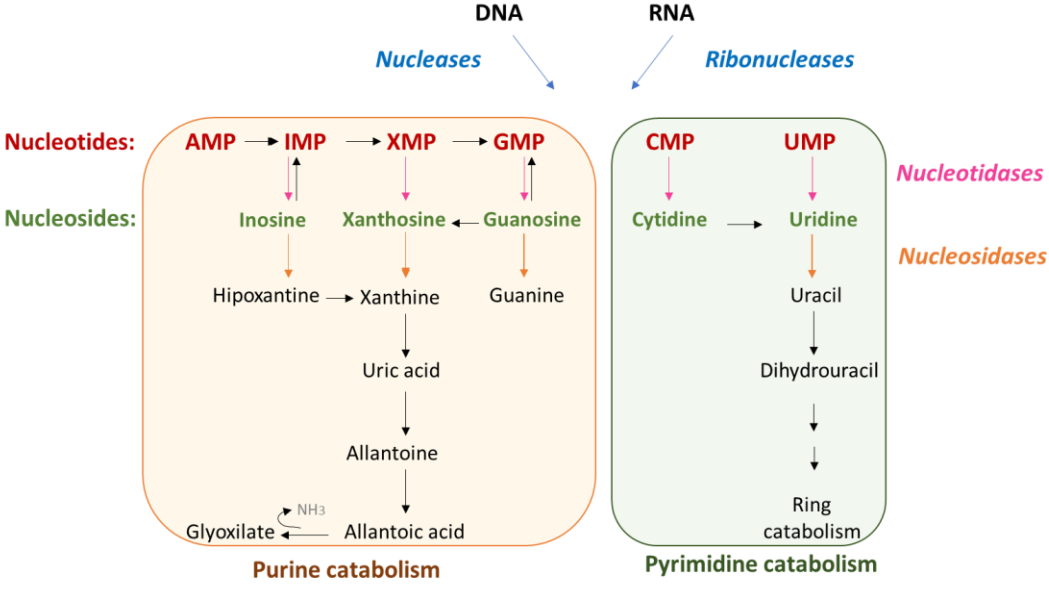

Figure 1. *Simplified overview of the plant catabolism of nucleic acids, following the information provided in Baccolini et al.,2019; Heineman et al., 2021 and Witte and Herde, 2020. The enzymes involved in the degradation of nucleic acids (bold black), nucleotides (brown) and nucleosides (green) are shown in blue, pink and orange, respectively.*

The main enzymatic activities involved until nucleobases formation are discussed below.

2.1. Enzymes involved in nucleic acids degradation.

Nucleic acids are macromolecules composed of linear polymers of nucleotides, linked by ester phosphate bonds. They can range in size from a few nucleotides to over billions nucleotide pairs in a eukaryotic chromosome (Minchin and Lodge, 2019). Both RNA and DNA have only two types of phosphodiester bonds for cleavage, either at the 5' position or the 3' position of a scissile phosphate. Nevertheless, the structures and catalytic mechanisms of RNA and DNA degrading enzymes are highly varied and complex (Mikkola et al., 2018). The cleavage of phosphodiester bonds is presumed to occur via a general acid-base catalysis, involving three stages: nucleophilic attack, the formation of a highly negatively charged penta-covalent intermediate, and the cleavage of the scissile bond (Yang, 2011).

The enzymes responsible for catalysing the hydrolysis of the phosphodiester bond in DNA are referred to as **nucleases**. These enzymes play a significant role maintaining the integrity of DNA by participating in processes such as recombination, repair, and replication (Symington, 2016). Based on their enzymatic properties, nucleases are classified into endonucleases or exonucleases, although the majority of identified nucleases so far are endonucleases (Sakamoto and Takami, 2014). Likewise, endonucleases are classified into two different classes. The first class is the Zn^{2+}-dependent endonucleases, characterized by its requirement for Zn^{2+} and an acidic optimum pH. This group of enzymes prefers to use ssDNA and RNA rather than dsDNA as substrates. The second class is the Ca^{2+}-dependent endonucleases, characterized by its dependence on Ca^{2+}, an optimal pH in the neutral range, and an active site similar to that of staphylococcal nucleases (Lesniewicz et al., 2012). In common bean, activities associated with nucleic acid degradation have been described in situations demanding significant nutrient mobilization, such as the development of embryonic axes (Lambert et al., 2014) and the senescence of cotyledons and leaves (Lambert et al., 2016; 2017).

The enzymes that catalyse the hydrolysis of the phosphodiester bond present in RNA are **ribonucleases**, an activity essential in RNA processing, maturation, and RNA interference (RNAi) (Yang, 2011). RNA is the main nucleic acid in most tissues and can function both as genetic material and as a catalytic entity (ribozymes). Like protein enzymes, ribozymes possess an active site that specifically binds to a substrate and facilitates its conversion into a product (Scott, 2007). A substantial number of RNases can catalyse the cleavage of phosphodiester bonds without the requirement for metal ions. This capability is largely attributed to the hydroxyl group at the 2' position of ribose, which readily engages in intramolecular nucleophilic attack on the adjacent 3' phosphate, thus disrupting the RNA backbone (Yang, 2011).

2.2. Enzymes involved in nucleotides dephosphorylation.

Nucleotides are monomers that make up nucleic acids, act as energy intermediates, and serve as precursors for β-class vitamins and essential coenzymes such as NAD, FAD, and SAM (Zrenner et al., 2006). The availability of nucleotides during the early post-germinative development of seedlings is a crucial factor for germination success (Stasolla et al., 2003). The regulation of nucleotide synthesis, recycling, and degradation during post-germinative development has been studied (Mohlmann et al., 2010). Nucleotides can be synthesized *de novo* or derived from nitrogenous bases or nucleosides originating from recycling pathways (Witte and Herde., 2020). It has been proposed that during the initial stages of seedling development, nucleotide synthesis takes place through recycling pathways involving compounds derived from nucleic acid degradation (Zrenner et al., 2006).

The dephosphorylation of nucleotides to nucleosides could potentially be catalysed by a nonspecific acid phosphatase or a specific nucleotidase (Bogan and Brenner, 2010). **Phosphatases** are enzymes that catalyse the hydrolysis of organic phosphorus compounds into inorganic forms accessible to plants (Dinkelaker and Marschner, 1992; Wang and Liu, 2018). They are categorized into acid phosphatases and alkaline phosphatases, depending on their optimal pH (Araujo and Vihko, 2013). Basic phosphatases are characterized by their strong

substrate specificity. Within the category of acid phosphatases, two subgroups can be identified: those lacking substrate specificity, which play a critical role in phosphate recycling and transport, and those displaying some degree of specificity, which participate in various metabolic functions (Duff et al., 1994). Acid phosphatases are responsible for catalysing the hydrolysis of a wide range of phosphorylated compounds, encompassing both natural and synthetic substances.

Nucleotidases are a type of phosphatase that specifically catalyse the dephosphorylation of nucleotides. In developing common bean seeds, a phosphatase belonging to the HAD superfamily with high affinity for nucleotides was successfully purified (Cabello-Diaz et al., 2012), and the gene encoding this enzyme was identified (Cabello-Diaz et al., 2015). Later, a second gene encoding nucleotides and with high expression in nodules, a tissue with high nucleotidase activity required for ureides synthesis, was characterized (Galvez-Valdivieso et al., 2020). Interestingly, these two enzymes were not inhibited by the inclusion of molybdate in the reaction mixture (Cabello-Diaz et al., 2015; Galvez-Valdivieso et al., 2020), a compound known to inhibit most acid phosphatase activities (Scheener et al., 2019 cited in Kavka et al., 2021). Recently, the whole HAD superfamily genes of common bean has been identified, and some of them showed induction of its expression after methyl jasmonate treatment (Galvez-Valdivieso et al., 2021).

2.3. Enzymes involved in nucleosides degradation.

Nucleoside hydrolases (NSH) are enzymes that catalyse the breakdown of nucleosides into ribose and nucleobases (Figure 1). Nucleosidases have been isolated from various sources, including bacteria, parasitic protozoa, plants, marine invertebrates, and baker's yeast, but not from mammals (Ashihara et al., 2018). While it is common for nucleosides and nucleobases to be salvaged into their respective nucleotides, plants possess a unique ability, distinct from many other organisms: They can completely degrade both purine and pyrimidine bases (Werner and Witte, 2011). These reactions facilitate the recycling of nitrogen contained within the heterocyclic nucleobases. Additionally, intermediates

formed during the degradation of purines, such as allantoin and uric acid, also play a role in mitigating oxidative stress (Witte and Herde, 2020). In contrast, intermediates from pyrimidine degradation are believed to be utilized by enzymes in specialized metabolic pathways, ultimately leading to the synthesis of specialized compounds (Ashihara et al., 2018).

3. Ribonucleases

Ribonucleases (RNases) are found in a wide range of organisms and play a crucial role in breaking down RNA. RNAses are essential for nucleotide metabolism, as they facilitate the hydrolysis of the 3'-5' phosphodiester bonds between adjacent nucleotides in various types of RNA, including single-stranded, double-stranded, and DNA-RNA hybrids (Luhtala and Parker, 2010).

Among ribonucleases, transferase-type RNases are a family of enzymes that catalyse the cleavage of single-stranded RNA through a 2'-3' cyclic phosphate intermediate, resulting in the production of mono- or oligonucleotides with a 3' terminal phosphate group (Deshpande and Shankar, 2002; Borniego and Innes, 2023). Based on their distribution, pH preferences, and base specificity, these RNases are categorized into three different families: RNases A, T1, and T2 (MacIntosh et al., 2011).

The **RNase A** family encompasses RNases that exhibit specificity for pyrimidine bases. These enzymes typically possess a molecular mass ranging from 13 to 14 kDa (Irie, 1999) and display a preference for either alkaline conditions (pH 7-8) or slightly acidic environments (pH 6.5-7) (Luhtala and Parker, 2010). This family constitutes a group of homologous proteins that have been isolated in numerous vertebrate species but not in invertebrates (Garnett and Raines, 2022). They are named as RNase A family based in the best-known member, the bovine RNase A (Marshal et al., 2008). Extensive research has been conducted on the RNase A family. With respect to their physiological functions, their roles are primarily recognized as digestive enzymes within and outside of cells, such as pancreatic RNases and salivary gland RNases in mammals (Cuchillo et al.,

2011). Furthermore, current studies are investigating the potential clinical applications of RNases A, particularly in the context of their role as anticancer agents (Mohamed et al., 2022).

On the other hand, proteins belonging to the **RNase T1** family are specific for guanylic acid (Glow et al., 2016). These proteins have an approximate molecular mass of 12 kDa, and their optimal pH ranges between 7 and 8. They are found in fungi and bacteria (Deshpande and Shankar, 2002). The physiological role of these proteins in the organisms that produce them is varied and may not always be associated with RNA fragmentation.

Finally, the **RNase T2** family, named due to their similarity to *Aspergillus oryzae* RNase T2 (MacIntosh et al., 2020), consists of RNases that typically cleave all four types of bases (Luhtala and Parker, 2010). This group includes RNases with an average molecular mass of around 25 kDa, which were originally classified as acidic RNases (Irie, 1999). However, they can exhibit activity across a wide range of pH values (MacIntosh et al., 2010). The RNase T2 family is ubiquitously found in all eukaryotes and appears to play crucial roles in various biological processes (reviewed in MacIntosh et al., 2020). The RNase T2 family, which includes representatives from viruses, bacteria, fungi, and eukaryotes, is particularly widespread There are three distinguishing characteristics that differentiate ribonucleases T2 from the RNase A and RNase T1 protein families. Firstly, ribonucleases T2 exhibit a broader distribution. Secondly, the optimal pH for the activity of most ribonucleases T2 falls within the range of 4-5, in contrast to the alkaline (pH 7-8) or mildly acidic (pH 6.5-7) activity of enzymes in the RNase T1 and RNase A families. Thirdly, ribonucleases belonging to the T2 family typically cleave all four bases, while those from the A family tend to be specific for pyrimidines, and those from the T1 family for guanosine (Luhtala and Parker, 2010).

Transferase-type ribonucleases can be also classified according to their pH as acidic or alkaline. Alkaline RNases comprise the T1 family and those RNases from the A family with a basic pH. On the other hand, acidic ribonucleases are categorized into two types: weakly acidic RNases A (pH 6.5-7) and the T2 family

with an optimal pH of 4-5, representing the true acidic RNases (Irie, 1999). Since RNAses A are primarily distributed in non-secretory organs, they have been termed **non-secretory acidic RNases.** Extensive research has been dedicated to specific ribonucleases involved in various cellular processes, including RNA processing, RNAi, and antiviral defence. Nevertheless, cells also produce a set of general ribonucleases that are typically secreted or targeted to membrane-bound compartments (Luhtala and Parker, 2010). In plants, most proteins belonging to the RNase T2 family are either secreted or targeted to intracellular compartments within the secretory pathway, including the endoplasmic reticulum, vacuoles, or lysosomes, making them **secretory acidic RNases** (MacIntosh et al., 2020). When they enter the secretory pathway, these proteins are usually glycosylated in eukaryotic cells. However, there are cases in which RNase T2 proteins enter the cytoplasm; for example, in budding yeast, the RNase T2 protein Rny1 is released from the vacuole into the cytosol during oxidative stress (Thompson and Parker, 2009).

4. RNases T2

Members of the RNase T2 enzyme family catalyse endonucleolytic cleavage of RNA through a 2'-3'-cyclic phosphate intermediate. To date, all isolated acid RNases belong to the RNase T2 family. These ribonucleases are the most widely distributed among most eukaryotes, being found in the genomes of protozoa, plants, animals, although they have also been found in many bacteria and some viruses (Borniego and Innes, 2023).

At least one member of the RNase T2 family has been found in every eukaryotic genome that has been sequenced, with trypanosomatids being the only exception (Shang et al.,2018; Fricker et al., 2019). In the genomes of most non-plant organisms, only a single highly conserved gene from this family is present, and it has been postulated that RNase A members have supplanted RNase T2 in various biological functions within these organisms (Hillwig et al., 2009; MacIntosh et al., 2011). Nevertheless, in the case of plants, there has been a notable prevalence of gene duplication events and substantial divergence among

RNase T2 variants (Igic and Kohn, 2001; Shouzheng et al., 2022). In fact, at least four RNase T2 genes have been identified in the genome of each seed-bearing plant that has been sequenced (Ramanauskas and Igic, 2017). The range of RNase T2 proteins has broadened, and individual proteins have evolved to serve various functions (MacIntosh et al., 2020). For instance, the RNase T2 family is composed of five members in Arabidopsis (Igic and Kohn, 2001), eight in rice (MacIntosh et al., 2010), thirteen in common bean (Diaz-Baena et al., 2020) and soybean (Azizkhani et al., 2021), and twenty-one in *Eucommia ulmoides* (Qing et al., 2021). This suggests that besides the ancestral function of RNases, these proteins have acquired a variety of functions in plants.

A wide range of biological functions has been proposed for these ribonucleases, including nucleic acid clearance, degradation of self-RNA, serving as extra- or intracellular cytotoxins, and modulation of host immune responses. Recently, members of the RNase T2 family have been implicated in human pathologies such as cancer and parasitic diseases (MacIntosh et al., 2011) Interestingly, certain functions of RNase T2 family members are independent of their nuclease activity (MacIntosh et al., 2010).

4.1. Mechanism of action and structure

The structure and RNA cleavage mechanism of ribonucleases T2 are well understood. Their enzymatic mechanism closely resembles that of other ribonuclease transferases, involving a two-step process of general acid-base catalysis (MacIntosh et al., 2020). In each of these steps, the involvement of one to three histidine residues is essential for facilitating the acid-base catalysis required for transphosphorylation reactions (Irie, 1999; Thorn et al., 2012).

One of the distinctive characteristics of these ribonucleases lies in the existence of two common sequences, denoted as CAS I and CAS II. These sequences are conserved from viral RNases to RNases of animal origin (Figure 2). Within these

segments, crucial amino acid residues essential for catalytic function, such as histidine and glutamate, are located (Luhtala and Parker, 2010).

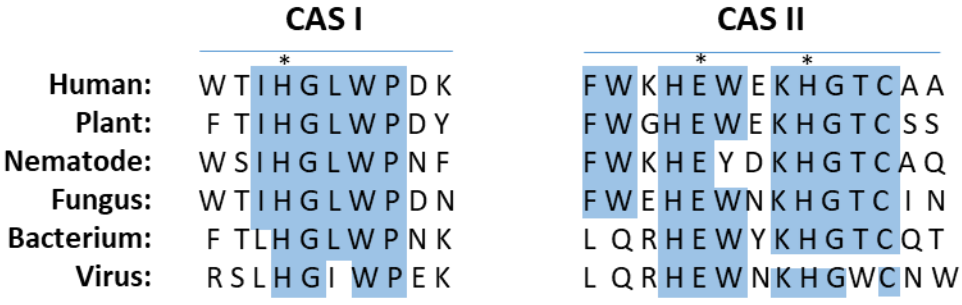

Figure 2. Amino acid sequences of the CAS I and CAS II domains essential for the activity in RNases T2 across different organisms*. CAS I and CAS II sequence of RNAse T2 are represented for human (O00584), plant (Arabidopsis thaliana; P42814), nematode (Caenorhabditis elegans; O61887), fungus (Aspergillus oryzae; P10281), bacteria (Vibrio aerogenes; A0A1M5ZPI9) and virus (classical swine fever virus; P19712). Next to the name of the individual, the access number of the Uniprot database (https://www.uniprot.org/) is indicated. The amino acids that match in at least 4 sequences are shown in blue. Histdine and glutamate essentials for catalytic function are marked by an asterisk.*

While the primary sequence identity among eukaryotic and prokaryotic enzymes is low, there are conserved secondary structures containing key hydrophobic residues associated with the active site of RNase T2 (reviewed in Wu et al., 2020). Fungal RNases have 10 half-cysteine residues, whereas animal and plant RNases have 8, with 4 of them being common to all RNases, including those of microorganisms. Hence, these 4 half-cysteine residues hold fundamental importance, and the other 4 can be substituted with different amino acids (Irie, 1999). Across all RNases, there are conserved sequences containing hydrophobic amino acids like leucine, isoleucine, phenylalanine, and tyrosine, which are likely crucial for maintaining an active conformation and facilitating mutual interactions within enzymatic molecules (Irie, 1999).

The crystal structure of T2 ribonucleases reveal the presence of a conserved central α/β secondary structure which closely resembles to the fungal ribonuclease (RNase Rh of *Rhizopus niveus*) (De Leeuw et al., 2007). All characterized members of the RNase T2 family contain a central antiparallel β-sheet with four strands (β1, β2, β4, and β5), a small antiparallel β-sheet with two strands (β3 and β7), and three α-helices (αB, αC, αD). The enzyme's catalytic site primarily resides in the β2 and β5 strands and the αC helix. The histidine residues present in β2 and αC, in conjunction with surrounding residues, collectively form the conserved active site motifs referred to as CAS I and CAS II. In several proteins, modifying these histidine residues through DNA mutation or chemical alterations can deactivate the RNase activity, both *in vitro* and *in vivo*. This ability to deactivate the catalytic capacity of these proteins provides a specific genetic mechanism for determining whether the functions of RNase T2 proteins are dependent on their catalytic capabilities (Thompson and Parker, 2009).

4.2. Classification and Functions of RNase T2 Ribonucleases

The plant members of the RNase T2 family are classified into two subfamilies, S-RNases and S-like RNases, and three classes (I-III). Class III RNases have been defined as S-RNases, while class I and II RNases are referred as S-like RNases (Ramanauskas and Igic, 2017; Azizkhani et al., 2021). The categorization of the RNase T2 family in plants into these three classes relies on phylogenetic analysis, sequence similarity, gene structure, and the number and location of introns.

S-RNases are encoded by the S genes and function as self-incompatibility factors, whereas S-like RNases are homologous to S-RNases but are not involved in self-incompatibility (Roalson and McCubbin, 2003; Fuji et al., 2016). S-like RNases are predicted to be involved in defence responses and metabolic processes. They are notably associated with phosphate scarcity, inhibiting hyphal growth, senescence, programmed cell death, and modulate response to pathogen (Rosenberg, 2008; Stigter and Plaxton, 2015; Sugawara et al., 2016; Gho et al., 2020).

To date, genes encoding **class I RNases** have been exclusively identified in plant species. They encode enzymes that play a role in various stress responses (MacIntosh et al., 2020). These genes exhibit evidence of gene duplications and gene arrangement, leading to substantial variability within this class. Consequently, the number of class I proteins varies among individual species (MacIntosh et al., 2020). Induction of these RNases has been observed in response to abiotic stress conditions, including salt stress and phosphate deficiency stress (Diaz-Baena et al., 2020; Gho et al., 2020).

In contrast, genes encoding **Class II RNases**, are usually present as a single copy per genome (MacIntosh et al., 2020), and are conserved across all eukaryotes. They play a crucial role in maintaining cellular homeostasis and are frequently expressed constitutively (Floyd et al., 2017). It is postulated that a pathway similar to ribophagy mediates the turnover of rRNA in normal, unstressed cells, which is essential for maintaining cellular homeostasis (MacIntosh et al., 2011). RNS2, the Class II enzyme found in *Arabidopsis thaliana*, is primarily located in the vacuole (Floyd et al., 2017). The investigation of *rns2* mutants has provided evidence for the enzyme's essential role in the regular turnover of rRNA. In the *rns2-2* null mutant, the half-life of both 28S and 18S rRNA nearly doubled (Hillwig et al., 2011), and rRNA accumulated within their vacuoles (Floyd et al., 2015). These findings indicate that rRNA renewal is a critical process for maintaining cellular homeostasis (Hillwig et al., 2011).

Regarding **Class III RNAses,** their only well-established function is a role in gametophytic self-incompatibility, which prevents self-pollination observed in many flowering plants (Rojas et al., 2015). However, other functions have also been assigned to this class (Rojas et al., 2015; 2018). Incompatibility results from the cytotoxic activity of S-RNases, while compatible pollen tubes avoid this enzyme (McClure et al., 2011). The absence of class III ribonucleases is a notable characteristic in the Arabidopsis genome (Ramanauskas and Igic, 2017).

Proteins belonging to class I and II are ubiquitous across all land plants, while class III proteins are exclusively found in eudicots (Ramanauskas and Igic, 2017). The widespread presence of RNase T2 enzymes in nearly all eukaryotic

genomes suggests their vital biological significance. Phylogenetic and gene expression studies suggest that the ancestral function of RNase T2 enzymes was related to homeostasis functions, associated with class II ribonucleases (MacIntosh et al., 2020).

Nutrient uptake in extracellular space is a potential function of ribonucleases T2. In plants, some T2 ribonucleases are activated under shortage of phosphate, and they might be involved in obtaining phosphates from nucleic acids. Thus, these proteins could break down nucleic acids, either inside or outside cells, to get essential nutrients (Melino et al., 2018). In plants, T2-type ribonucleases like LX and LE in tomato, as well as RNS1 and RNS2 in Arabidopsis, become more active under phosphate deficiency, supporting a role of these enzymes in RNA recycling (Bariola et al., 1994; Kaleta et al., 1998). Additionally, carnivorous plants have these ribonucleases highly active in their digestive organs, possibly to get phosphate and nitrogen from the prey they catch as part of their carnivorous lifestyle (Nishimura et al., 2013).

It has been proposed that ribonucleases T2 play diverse functions across various organisms which are independent of RNA hydrolysis capacity. As a result, there has been a growing interest in the study of these enzymes because their cytotoxic capability may be related to a potential defensive function (Yue et al., 2023).

In humans, there is only one member of RNase T2, and its implications for human health are being investigated in various fields of medicine and biology. RNase T2 has been observed to induce apoptosis in cancer cells by degrading essential cellular RNA, suggesting its potential for the development of cancer-targeted therapies (Acquati et al., 2019). Furthermore, RNase T2 has been studied in the context of neurodegenerative diseases such as amyotrophic lateral sclerosis (Hamilton et al., 2020). Alterations in RNase T2 regulation could be related to the accumulation of abnormal RNA in certain neurodegenerative conditions. Humans lacking RNase T2 exhibit defects in neurological development, possibly due to aberrant immune system control (Henneke et al., 2009; Kameli et al., 2019). In addition, the regulation of RNase T2 could be involved in autoimmune diseases such as systemic lupus erythematosus (Zhu et

al., 2022) and rheumatoid arthritis (Angeli et al., 2023). These diseases are characterized by a hyperactive and abnormal immune response. Since RNases T2 selectively degrade RNA, its potential use in gene therapy has also been explored. Thus, they could be employed to modulate the expression of specific genes, which could be useful in the treatment of genetic diseases or in the regulation of gene expression in various medical applications (Hamilton et al., 2020). Then, a better knowledge of RNases T2 could clarify the mechanisms of these diseases and provide new therapeutic avenues.

5. Common bean T2 ribonucleases

We have recently described the RNAse T2 family in common bean. It consists of 13 members which were named from PvRNS1 to PvRNS13 (Diaz-Baena et al., 2020). A phylogenetic analysis with the 5 RNases T2 from Arabidopsis (Igic and Kohn, 2001) allowed the classification of common bean RNases T2 into three classes (Figure 3). The common bean PvRNS1, PvRNS2, PvRNS3 and the Arabidopsis AtRNS1, AtRNS3, AtRNS4 and AtRNA5 belong to the Class I, PvRNS4 and AtRNS2 to Class II, while PvRNS5 to PvRNS13 are grouped in Class III, which has no member in Arabidopsis (Figure 3). Therefore, S-like RNases T2 in common bean is composed of PvRNS1 to PvRNS4, whereas the others 9 proteins are classified as S RNases T2 (Diaz-Baena et al. 2020).

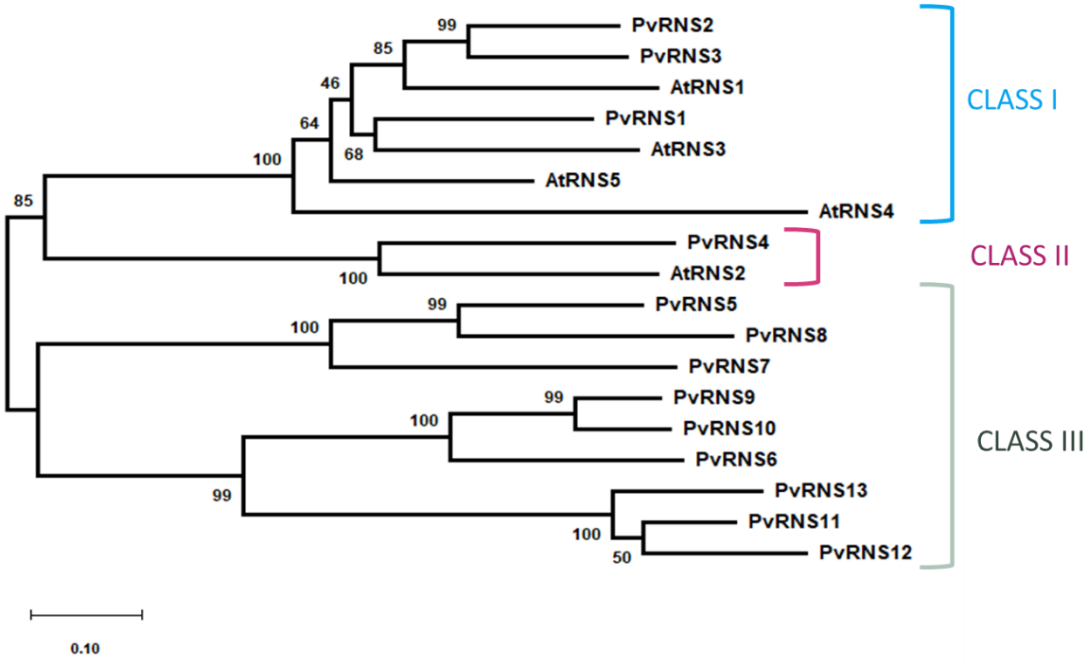

Figure 3. Evolutionary relationships of T2 ribonuclease sequences of common bean and Arabidopsis. *The phylogenetic tree was constructed with the sequences of the T2 ribonucleases found in common bean and Arabidopsis using the programme MEGA-X v.11 and the neighbour-joining model. The evolutionary distance was calculated using the Poisson correction method. The numbers at the nodes represent the bootstrap values with 1,000 replicates. The tree is accurately depicted in terms of proportions, where branch lengths are in the same measurements as those used to deduce the phylogenetic tree's evolutionary distances. These distances are expressed as the number of amino acid substitutions per site.*

In a previous study, we showed that the common bean RNases T2 are categorized as soluble proteins and most of them, with the exception of PvRNS2 and PvRNS4, are expected to be found in the extracellular space based on deduced sequence for the predicted protein (Diaz-Baena et al., 2020).

Common bean RNases T2 genes exhibited the CAS I and CAS II domains conserving the two histidines essential for its activity (Figure 4). The importance of these histidines has been functionally demonstrated in Arabidopsis (Megel et

al., 2019). The identity of the predicted sequences for CAS I and CAS II domains are very high among common bean RNAses T2 and Arabidopsis RNAses T2, suggesting that all the genes encode functional ribonucleases.

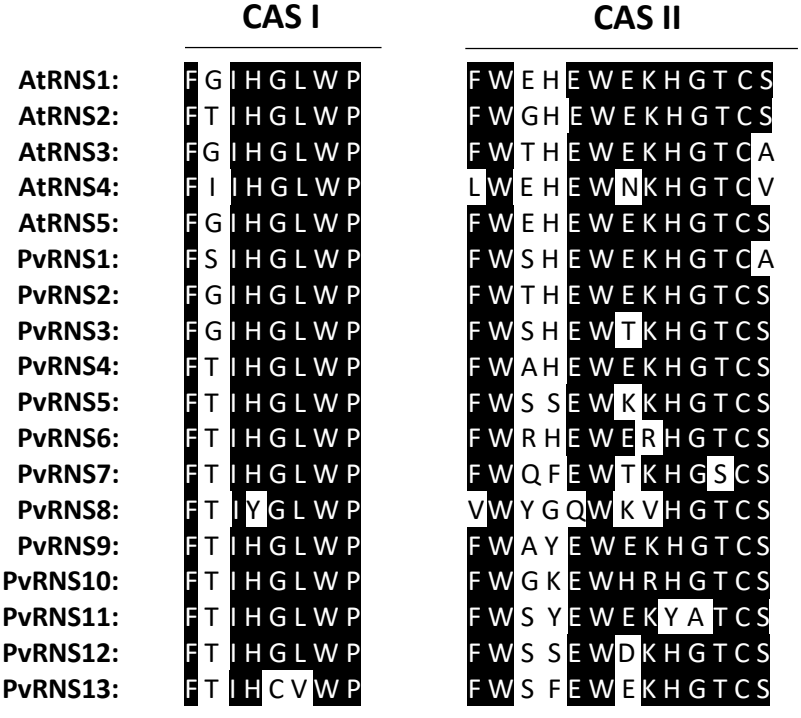

Figure 4. Alignment of CAS I and CAS II domains from T2 ribonucleases identified in common bean and Arabidopsis thaliana. *Arabidopsis and common bean sequences were obtained from the Phytozome database (https://phytozome-next.jgi.doe.gov/). Common bean ribonucleases were named as previously in Díaz-Baena et al. (2020). The alignment of the aminoacidic sequences was performed by the MUSCLE method from MEGA-X v.11. The amino acids that match in at least 9 sequences are shown in black*

The expression of S-like RNAses of common bean has been analysed in seedlings exposed to salt stress which demonstrated the specific induction of *PvRNS3* in radicles (Diaz-Baena et al., 2020). This suggests a putative role of S-like RNases in situations that involve a significant mobilization of nutrients for the plant, making interesting their study

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2. HYPOTHESIS / OBJECTIVE

HYPOTHESIS

During situations of high energy demand, such as tissue growth and development or exposure to adverse environmental conditions or the presence of pathogens, plants mobilise their resources to ensure the survival and success of the plant in its environment. Nucleic acids are relatively abundant compounds that can be broken down in essential elements, such as nucleotides and nitrogenous bases and other molecules, which are required for the synthesis of proteins, enzymes, and cofactors. Therefore, the hypothesis of this work is that nucleic acids can play an important role supplying essential nutrients in conditions of high nutrient mobilization.

OBJECTIVES

The main objective of the thesis is to determine the role of nucleic acids in situations of high nutrient mobilization. To this end, the following specific objectives have been addressed:

1. To analyse the activity and expression level of common bean T2 S-Like ribonucleases during cotyledon senescence, a process with high nutrient mobilisation.

2. To analyse the activity and expression level of nucleic acid degrading activities such as nucleases and ribonucleases, as well as nucleotidases, nucleosidases and allantoinases during seed filling stage.

3. To analyse the role of T2 S-like ribonucleases in the seed coat.

4. To purify and characterise the protein encoded by *PvRNS3*, a ribonuclease gene with high expression in some abiotic stress situations.

3. CHAPTERS

CHAPTER I:

S-LIKE RIBONUCLEASE T2 GENES ARE INDUCED DURING MOBILISATION OF NUTRIENTS IN COTYLEDONS FROM COMMON BEAN

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ABSTRACT

Germination and seedling development are crucial phases in a plant's life cycle with economical and agronomical implications. The RNA quality in seeds is linked to seed viability, being an important agronomic trait since this leads to a loss in germination efficiency. In addition, RNA can be an important phosphorous reservoir in seeds, affecting the efficiency of the mobilisation of nutrients towards the seedlings. However, knowledge about the physiological function of ribonucleases during germination and seedling development is scarce. We analysed the ribonuclease activities of cotyledons during these processes and the expression of S-like ribonucleases T2. Ribonuclease activity was detected in cotyledons at 1 day after imbibition and the specific activity increased during germination and seedling development, reaching a maximal value at 10 days after imbibition. At this stage, the levels of proteins and RNA in cotyledons were very low. Using in-gel assays, three ribonucleases were detected with apparent molecular masses of 16, 17 and 19 kDa along cotyledon ontogeny. The S-like ribonucleases T2 family consists of four genes in common bean (*PvRNS1* to *PvRNS4*). The expression of *PvRNS1*, *PvRNS2* and *PvRNS4* increased in the phase of nutrient mobilisation in cotyledons. The expression of PvRNS1 increased 1000 fold in cotyledons, from 1 to 6 days after imbibition. The suppression of the induction of ribonuclease activity and gene expression in decapitated seedlings suggests that the regulatory signal comes from the developing axes. These results clearly state that S-like ribonucleases T2 are involved in RNA turnover in cotyledons during seedling development.

Keywords: ribonuclease; Phaseolus vulgaris; seedlings; cotyledons; nutrient mobilisation; RNA

1. INTRODUCTION

Seed germination and post-germinative growth are two crucial phases in the life cycle of higher plants. The development of the new plant depends on the reserves accumulated in seeds during their formation, having to reach photoautotrophism and at the end of these periods before their nutrient reserves become exhausted. Therefore, these two processes involve high mobilisation of nutrients. Cotyledons are the storage organs in most dicotyledonous seeds, their main function of which is to provide nutrients to the developing axes until the seedlings become photosynthetic. Cotyledons from epigeously germinating seeds, such as common bean, follow several stages during germination and seedling development. After early embryonic axes development, cotyledons go through a photosynthetic phase, ending with senescence and nutrients exhaustion. Therefore, the cotyledons provide an excellent experimental system to analyse the changes occurring during nutrient mobilisation, given their role as nutrient supplier.

Germination sensu stricto starts with the imbibition of dry seeds and finishes with the radicle protrusion through the seed coat (Rajjou et al., 2012). During this phase, both unbound metabolites and those obtained by the degradation of stored starch, proteins and oils in the embryo can provide the energy and nutrients needed for embryo growth and radicle protrusion (Rosental et al., 2014).

During germination, the metabolism needs to be reactivated in a dense and oxygen-poor-environment which involves changes in metabolic activities (Rosental et al., 2014). The mobilisation of nitrogen and phosphorous during germination is a process poorly understood in comparison with carbon, despite the high demand for these compounds by the developing axes to actively synthesize proteins and nucleic acids. In common bean, the main globulin is phaseolin, representing 34-50 % of total protein in mature seed (Harada et al., 2010). No differences in globulins were observed in common bean cotyledons during germination, whereas they became exhausted in embryonic axes before radicle protrusion (Lambert et al., 2016). However, during early seedling

development, from day 4 to day 7 after imbibition, phaseolin content in cotyledons suffers a huge decrease, becoming exhausted in cotyledons at 11 days after imbibition (Lambert et al., 2016). Coincident with the degradation of phaseolin, enzymes involved in the degradation of ssDNA are induced (Lambert et al., 2016), as well as allantoinase, an enzyme that catalyses the degradation of the ureide allantoin, a reaction product of the catabolism of purine nucleotides (Quiles et al., 2009).

Nucleic acids are relatively abundant organic compounds in plant tissues. In addition to carbon, they contain nitrogen and phosphorus and, therefore, could play an important role as reservoirs for these compounds. Nucleic acids constitute the largest organic phosphorus reservoir in plants (Veneklaas et al., 2012), RNA being the most abundant, since it represents around 85 % of nucleic acids and 47 % of organic phosphorus (Veneklaas et al., 2012). Due to the fact of this abundance, RNA could be crucial as a phosphorous supply in situations of high nutrient mobilisation, as RNA is present in dried seeds and can be transiently used for macromolecule synthesis during early phases of germination (Zhao et al., 2020). In addition, the integrity of this stored RNA is indicative of seed age and could be a good parameter to determine seed germination capacity (Fleming et al., 2019; Zhao et al., 2020). The time span during which seeds remain viable or seed longevity is an important trait for agronomy, and the reduction of seed longevity has been attributed to oxidation of nucleic acids, in addition to lipids and proteins (Sano et al.,2020). Stored RNA has a crucial role during gemination, which can proceed without the synthesis of new mRNAs, whereas it is arrested by the inhibition of translation (Rajjou et al., 2004; Sano et al., 2012). The regulatory mechanisms that control the translation of the stored mRNA during germination has been recently reviewed (Sano et al., 2020). This stored RNA is replaced as the germination progresses and cells start to use new transcripts for protein synthesis (Bewley, 1997). During the mobilisation phase, nucleic acids stored in cotyledons are degraded, and the resulting products might function as the P and N source for the developing seedlings. Despite this, knowledge about nucleic acid degradation in senescing cotyledons is very scarce (Lambert et al., 2016).

Ribonucleases are the enzymes involved in RNA degradation, participating in a wide range of processes (Zheng et al., 2014). Due to the relative abundance of ribonucleic acid mentioned above, these enzymes could play a relevant role in physiological situations with high mobilisation of nutrients. Ribonucleases belonging to the T2 family are the most widely distributed and have been described in bacteria, fungi and eukaryotes (Luhtala et al., 2010). In medicine, these enzymes have become of enormous interest because they seem to play a critical role in the pathophysiology of inflammation and cancer (Wu et al., 2020). In plants, the biological role of S-like ribonucleases is poorly understood with suggested roles during Pi deficiency and in defence and stress responses, among others (MacIntosh et al., 2020). Members of this family have been described in Arabidopsis (Igic and Kohn, 2001), rice (MacIntosh et al., 2010) and common bean (Diaz-Baena et al., 2020). The plant RNase T2 family is divided into two subfamilies, S-RNases and S-like RNases (Green, 1994). The S-RNases are involved in gametophytic self-incompatibility, whereas the S-like RNases participate in a variety of processes including defence against biotic or abiotic stresses, salvage pathways and production of tRNA-derived small RNAs (MacIntosh et al., 2020). The RNase T2 family members are also classified into three classes (I to III) (Ramanauskas and Igic, 2017); Class III includes the S-RNases and the Classes I and II the S-like RNases. Most of the RNase T2 family proteins are either secreted or targeted to cellular compartments of the secretory pathway (MacIntosh et al., 2020).

In common bean, nuclease activities have been described during embryonic axes development (Lambert et al., 2014) and cotyledons and leaf senescence (Lambert et al., 2016; Lambert et al., 2017), processes that requires high mobilisation of nutrients. Recently, the ribonuclease T2 family of *Phaseolus vulgaris* was described, and it was found that one of its members, *PvRNS3*, is induced in radicles of seedlings exposed to salt stress (Diaz-Baena et al., 2020). In this study, we assessed the changes in ribonuclease activities and in the expression of S-like ribonucleases T2 in cotyledons of common bean, examining the processes of germination and reserve mobilisation. We demonstrated that the expression of three genes coding for S-like T2 ribonucleases is induced in

common bean cotyledons during the phase of high nutrient mobilisation to the developing seedlings. This study will contribute to the understanding of the role of nucleic acids under nutrient mobilisation situations in common bean, the most important legume for human consumption (Broughton et al., 2003).

2. MATERIALS AND METHODS

2.1. Plant material

Common bean (*Phaseolus vulgaris*) seeds were sterilised by soaking in ethanol (30 s) and 0.2 % (*w/v*) sodium hypochlorite (5 min) and washing repeatedly with distilled water. Seeds were placed in Petri dishes (120 mm diameter) with wet filter paper, and seeds were allowed to germinate in the growth chamber. Distilled water was added routinely to maintain humidity.

To obtain the material from cotyledons during germination and seedling development, the seedlings were maintained in Petri dishes until 6 days after start of imbibition (DAI). At that moment, seedlings were transferred to pots containing vermiculite: perlite (3:1, *v/v*) and watered with distilled water until 10 DAI.

Decapitated seedlings were obtained from 3 DAI seedlings by cutting the epicotyls with a razor blade just above the junction of the cotyledon and the embryonic axis. These decapitated seedlings, obtained by epicotyl removal, were maintained in wet Petri dishes for 3 additional days. Cotyledons were obtained from the decapitated seedlings (6-D) and from 3 and 6 DAI whole seedlings as controls.

Cotyledons from seedlings developed in the presence of nutrients were obtained by watering with the nutrient solution described in Galvez-Valdivieso et al. (2013), enriched with 10 mM KNO_3 during germination and early seedling development. The collected plant materials were snap-frozen with liquid nitrogen and then stored in a −80 °C freezer until further analysis.

2.2. Preparation of Crude Extracts

Frozen plant materials were ground to a fine powder with mortar and pestle in liquid nitrogen and stored at -80 °C. Crude extracts were obtained from frozen powder by homogenisation with extraction buffer (50 mM TES buffer (pH 7.0) containing 0.15 % (*w/v*) sodium deoxycholate using 4 mL of buffer per gram of powder. After centrifugation at 15000 *g* for 10 min at 4 °C, the supernatants were transferred to new tubes and considered as crude extracts.

2.3. Total Soluble Protein Determination

The soluble protein concentration was estimated by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard and a Bio-Rad system.

2.4. Determination of Enzymatic Activities

Ribonuclease activity in agarose gels: Total RNA (1 µg) isolated from common bean radicles was incubated in a total volume of 10 µL with crude extracts from common bean cotyledons in 50 mM acetate (pH 5.5) at 37 °C. At times 0 and 10 min, 0.33 µL of MOPS buffer pH 7 (200 mM MOPS, 50 mM sodium acetate, 1 mM $EDTANa_2$) and 0.6 µL of formaldehyde were added to each tube and mixed. The reaction products were size fractionated on a denaturing formaldehyde agarose gel.

In-gel ribonuclease assays: Ribonuclease activity was determined as previously described by Diaz-Baena et al. (2020). Briefly, the gels contained RNA from torula yeast (0.75 mg mL^{-1}, Sigma-Aldrich). The crude extracts were heated for 10 min at 65 °C in sample buffer before loading the gels. After electrophoresis, gels were incubated twice for 10 min at room temperature in 10 mM of the indicated buffers containing 25 % (*v/v*) 2-propanol, and two additional times for 10 min at 4 °C in the same buffer without 2-propanol at 4 °C. The enzymatic reaction was developed at 50 °C in the same buffers at 100 mM for a variable time (15-40 min). The gels were stained with toluidine blue O (0.2 %, Sigma-Aldrich, Madrid, Spain) for 5 min and washed repeatedly with distilled water. Gels were photographed and the images inverted.

2.5. RNA Isolation

Total RNA was extracted from 50 mg of powdered tissue using the NZYol Reagent (NZYTECH, Lisbon, Portugal) following the manufacturer's instructions but including an additional LiCl precipitation step at the end of the procedure to improve the RNA quality. The RNA was finally dissolved in 20 µL of water. The RNA was visualised using denaturing formaldehyde agarose gel. The RNA concentration was determined using a nanoVue Plus Spectrophotometer (GE Healthcare, Little Chalfont, UK).

2.6. cDNA Synthesis

Two µg of total RNA were treated with RNAase-free DNAseI (NEB) to remove any traces of DNA. After treatment, the absence of contaminating genomic DNA was assessed by PCR. First strand cDNA was synthesised by reverse transcription of 2 µg of total RNA using RevertAid reverse transcriptase (ThermoFisher, Madrid, Spain) with random hexamer primers.

2.7. qRT

Quantitative real-time PCR was performed as indicated previously (Diaz-Baena et al., 2020). Primers for the four ribonuclease T2 and the two reference (actin and ubiquitin) genes were the same as described previously (Diaz Baena et al., 2020). At least three biological and three technical replicates were used for each experiment.

2.8. Statistics

All results are means of at least three independent experiments with at least two technical replicates. Values are mean ± SE. Statistical analyses were performed with SPSS Statistics, version 25. Significant differences according to Tukey's test are indicated with different letters ($p \leq 0.05$). A Student's t-test was performed comparing nutritive solution imbibition with control seedlings.

3. RESULTS

3.1. Total RNA and Protein during Cotyledon Development in Common Bean

Total RNA and protein were analysed in cotyledons from common bean during germination (1 and 3 DAI), early seedling development (3 and 6 DAI) and seedling establishment and cotyledon senescence (6 and 10 DAI). The level of RNA was determined spectrophotometrically and diminished during these stages with the biggest reduction from 6 to 10 DAI (Figure 1A). The level of RNA was monitored by electrophoresis of total RNA in agarose gels (Figure 1B). During germination, the level of total rRNA remained unchanged, decreased at 6 DAI and it became almost undetectable in senescing cotyledons at 10 DAI (Figure 1B). The level of total soluble protein in the same crude extracts followed a pattern very similar to that of total RNA (Figure 1C).

3.2. Ribonuclease Activities in Cotyledons of Common Bean

Ribonuclease activity in cotyledons from common bean was determined using total RNA isolated from common bean radicles as substrate (Figure 2) following the degradation of RNA by electrophoresis. Ribonuclease activity was detected in crude extracts from cotyledons at 1 DAI, and the specific activity increased during seedling development reaching maximal RNA degradation at 10 DAI (Figure 2). A similar pattern of activity was obtained when the activity was normalised using the same amount of fresh weight per assay instead of total soluble protein (Figure S1).

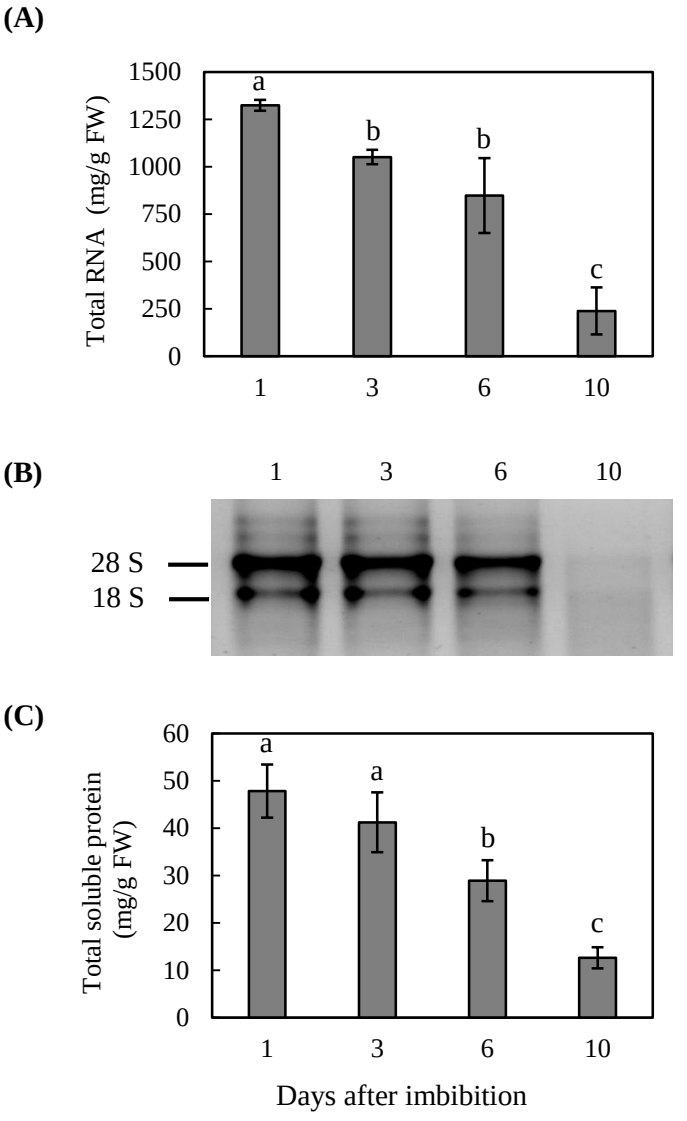

Figure 1. *RNA and protein in cotyledons from common bean during germination and seedling development stages. (A) Total RNA was extracted from 50 mg of powdered cotyledons collected at the indicated time after imbibition and resuspended in a final volume of 20 µL. The ribonucleic acid in each sample was quantified using spectrophotometer. (B) 1 µL of total RNA was loaded on agarose gels and separated by electrophoresis. (C) Total soluble protein was determined in crude extracts obtained from the former cotyledons. Values are mean ± SE of three independent experiments. Significant differences, according to Tukey's test, are indicated with different letters ($p \leq 0.05$).*

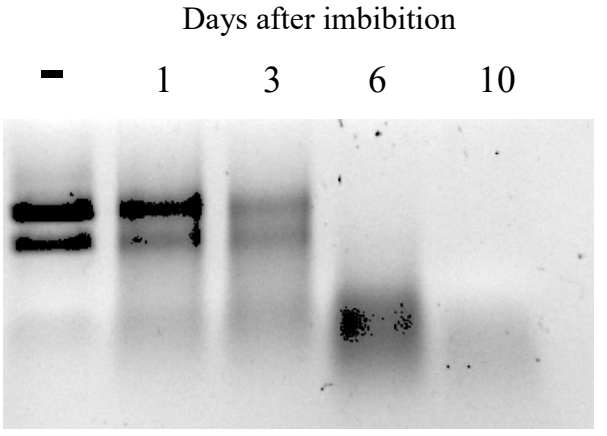

***Figure 2**. Ribonuclease activity in common bean during cotyledon development. Ribonuclease activity was determined in crude extracts obtained from cotyledons at the indicated days after start of imbibition (DAI) using 1 µg of total RNA isolated from common bean radicles as substrates and equal amounts of total soluble protein in crude extracts. As control, the total RNA used as substrate was incubated in the absence of crude extracts (−). After enzymatic reactions, the remaining rRNA was visualised in agarose gels after electrophoresis.*

The ribonuclease activity was determined as well by in-gel assays with total RNA from torula yeast (Figure 3). In this assay, the activity was determined after electrophoresis in gels imbibed with RNA and provides information regarding the relative mobility of proteins with ribonuclease activity. In this case, the assay was performed loading crude extract obtained from the same amount of fresh weight per lane to avoid the interference of reserve protein degradation during the process. The activity was determined at two pH conditions to differentiate between acidic and neutral enzymes, following the same experimental procedure previously carried out for nuclease activities in common bean cotyledons (Lambert et al. 2016). Three ribonuclease activities were determined in cotyledons at 1 DAI with a relative mobility in semi-denaturing conditions of 16, 17 and 19 kDa. Proteins with 17 and 19 kDa showed similar activity in acetate (pH 5.5) and Tris-HCl (pH 7) buffer, and their activity increased during seedling development (Figure 3). However, the 16 kDa enzyme showed higher activity at

pH 5.5 than 7.0, and its activity decreased in cotyledons after germination (Figure 3).

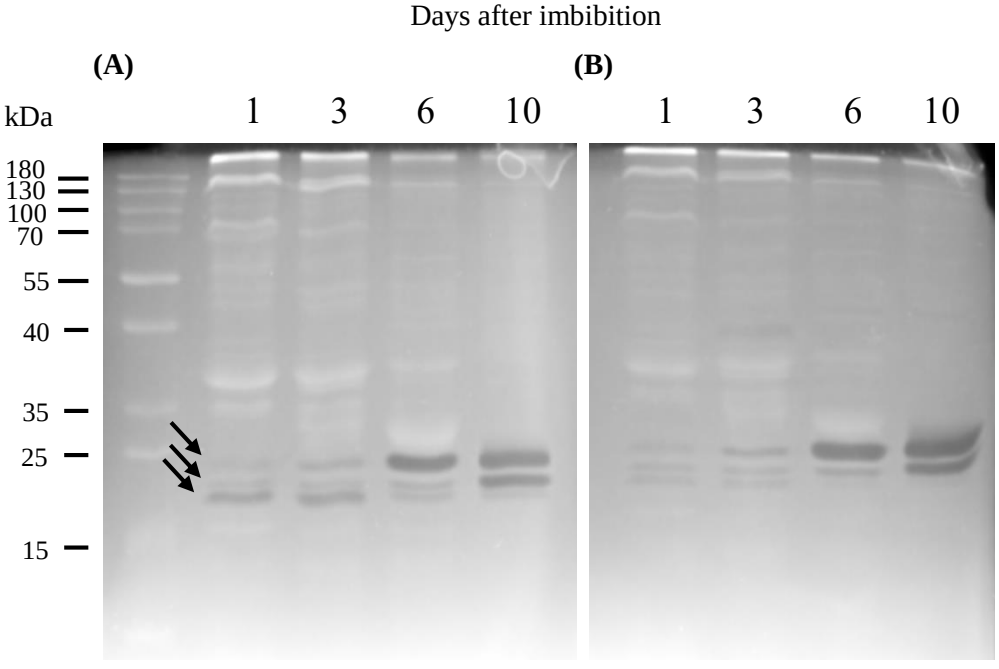

Figure 3. *In-gel ribonuclease activity in common bean during cotyledon development. Ribonuclease activity was determined in acetate buffer pH 5.5 (A) and Tris buffer pH 7 (B) using crude extracts obtained from cotyledons at the indicated DAI. Equal amounts of crude extracts were loaded per lane. The mobility of molecular mass markers is shown on the left. After enzymatic assays, the gels were photographed, and the images inverted. The main detected ribonucleases are marked with black arrows.*

3.3. S-Like Ribonuclease Genes Expression in Cotyledons of Common Bean

The expression levels of T2 ribonucleases from common bean belonging to Class I and II (*PvRNS1* to *PvRNS4*) were analysed in cotyledons during the germination and seedling development stages. The transcript levels of all the four genes were very low in cotyledons at 1 DAI and almost undetectable for *PvRNS1*, *PvRNS2* and *PvRNS3* (Figure 4). The level of *PvRNS1* remained low at 3 DAI and increased significantly at 6 and 10 DAI, whereas expression of *PvRNS2*

increased through seedling development, although the increase was less marked than that of *PvRNS1* (Figure 4). The level of expression of *PvRNS3* remained relatively low and without any significant change through these stages (Figure 4). The expression of *PvRNS4* progressively increased at 3 and 6 DAI and remained high at 10 DAI (Figure 4). The maximal expression of PvRNS1 and *PvRNS4* was at 6 DAI, whereas that of *PvRNS2* was at 10 DAI, with an increase in their transcript level of 970, 32 and 17 folds, respectively, in relation with the expression at 1 DAI.

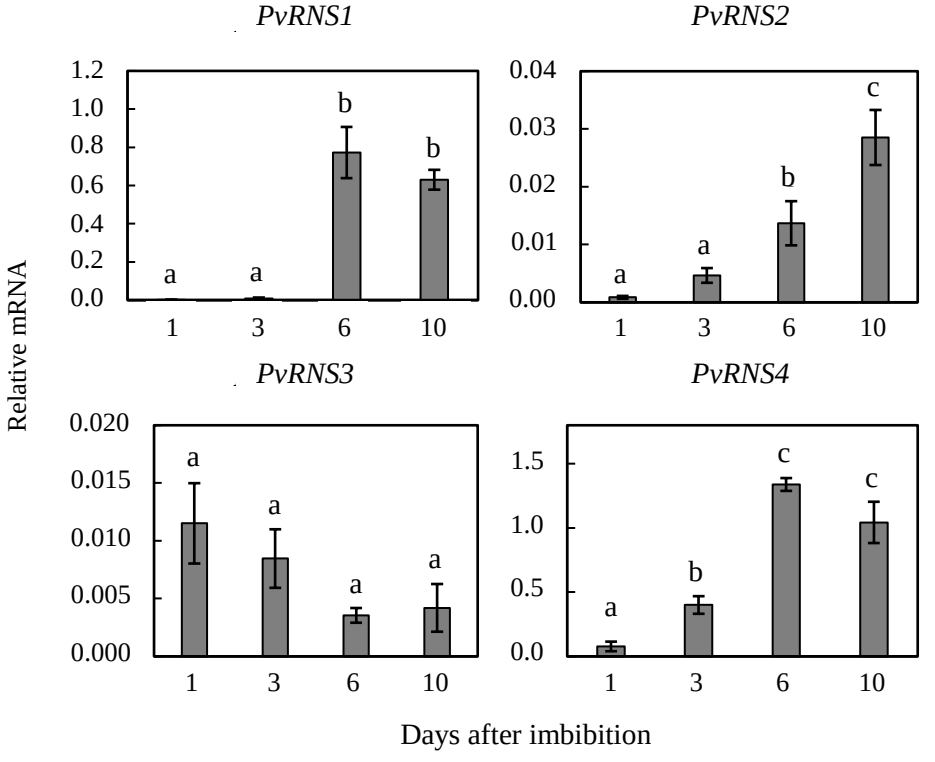

Figure 4. *Expression pattern of PvNRS1 to PvRNS4 in cotyledons during germination and seedling development. S-like T2 ribonuclease expression analysis was performed using qRT-PCR on total RNA samples extracted from cotyledons of common bean at the indicated DAI. The relative expression level was normalised using the geometric mean of two reference genes and analysed using the* $2^{-\Delta\Delta CT}$ *method. Values are mean ± SE of four independent experiments with three technical replicates per experiment. Significant differences, according to Tukey's test, are indicated with different letters (*$p \leq 0.05$*).*

To test if the induction of these genes required the presence of the developing axes either as sinks demanding nutrients from cotyledons or as receptors of a signal derived from the expanding axes, the expression of the former genes and the ribonuclease activity were determined in 3 DAI decapitated seedlings obtained after removing the epicotyl of seedlings just above the junction of the cotyledons and the embryonic axes. The decapitated seedlings without epicotyls were maintained in the growth chamber for three additional days and cotyledons were isolated from these seedlings (6-D) as well that from normal developed seedlings at 3 (3) and 6 DAI (6) (Figure 5). In this situation, the increase of the 19 kDa ribonuclease activity as well as the expression of *PvRNS1*, *PvRNS2* and *PvRNS4* were inhibited (Figure 5).

In the experiments presented above, seedling moisture was maintained with water to study the mobilisation of nutrients from the cotyledons. To determine if the response is due to the fact of stress by nutrient deficiency, we analysed the ribonuclease activities and the expression of S-like T2 ribonucleases in cotyledons from seedlings developed in the presence of nutrients (Figure 6). The availability of nutrients in the imbibition medium did not affect the activity of the three ribonucleases (Figure 6A, B) or the levels of transcript of the 4 S-like ribonucleases T2 (Figure 6C).

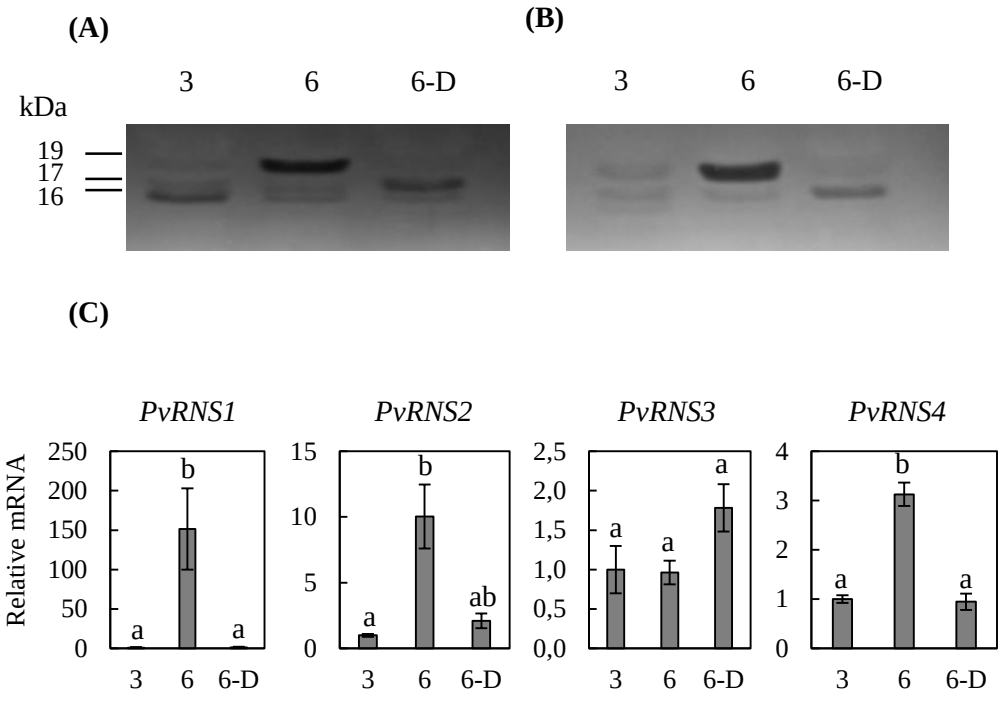

Figure 5. *Ribonuclease activity and expression of S-like T2 ribonuclease genes in cotyledons from decapitated seedlings. Three days after imbibition, seedlings were decapitated by removing the epicotyls and placed in Petri dishes for three additional days (6-D). As controls, cotyledons from seedlings at 3 (3) and 6 (6) DAI were collected. Ribonuclease was determined using the same amount of crude extract per lane in acetate buffer pH 5.5 (A) and Tris buffer pH 7 (B). S-like T2 ribonuclease expression was determined by qRT-PCR and the data were normalised against the expression at 3 DAI for each gene (C). Values are mean ± SE of three independent experiments with three technical replicates per experiment. For each gene, the values were normalised to the values in cotyledons at 3 DAI. Significant differences, according to Tukey's test, are indicated with different letters ($p \leq 0.05$).*

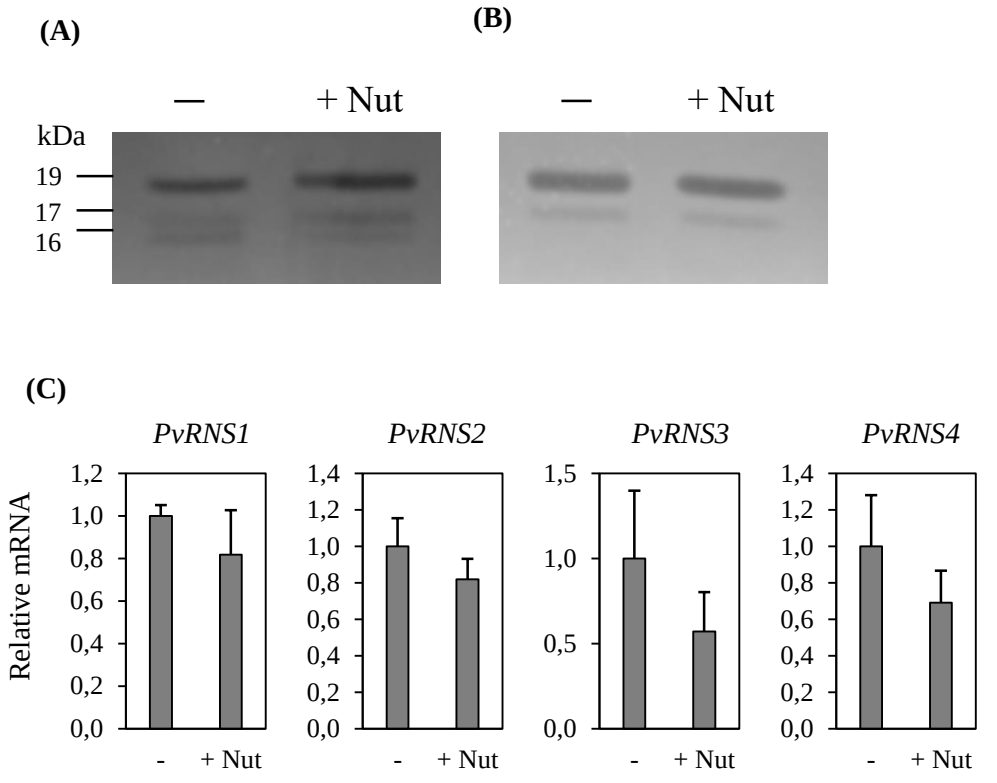

Figure 6. *Ribonuclease activity and expression of S-like T2 ribonuclease genes in cotyledons from seedlings developed in the absence (−) or presence of nutrient solution (+Nut). Ribonuclease was determined using the same amount of crude extract per lane in acetate buffer pH 5.5 (A) and Tris buffer pH 7 (B). S-like T2 ribonuclease expression was determined by qRT-PCR (C). Values are mean ± SE of four independent experiments with three technical replicates per experiment. For each gene, the values were normalised to the values in cotyledons from seedlings developed in water (−). Significant differences, according to Tukey's test, are indicated with different letters ($p \leq 0.05$).*

4. DISCUSSION

In this paper, we have shown that different ribonucleases are induced in cotyledons during early seedling development, particularly three that belong to the S-like ribonucleases T2 family and, that this induction occurred in the nutrient mobilisation phase.

The mobilisation of nutrients during seedling development is a crucial agronomic challenge for correct plant establishment. The involvement of the RNase T2 in

the degradation of RNA during this process has been poorly studied, although RNA is an important reservoir of organic N and P in plant cells (Veneklaas et al., 2012). Under nutrient starvation, RNA degradation could be a source for these elements. Thus, RNA yields are reduced in wheat under N starvation at the same time that a ribonuclease gene is upregulated, suggesting that RNA may be an important source of nutrients (Melino et al., 2018). Likewise, in *Saccharomyces cerevisiae*, a ribonuclease T2 is majorly involved in RNA degradation under N starvation conditions (Huang et al., 2015). Here, we described that ribonuclease activity in common bean increased in cotyledons, and we identified several ribonucleases in cotyledons.

Recently, we determined that *PvRNS3* is induced in radicles subjected to salt stress (Diaz-Baena et al., 2020). However, this is the only gene that does not change significantly in cotyledons during germination and seedling development stages, whereas the other three S-like T2 ribonuclease genes, *PvRNS1*, *PvRNS2* and *PvRNS4*, alter their expression in cotyledons during seedling development. Although, it is very probable that these three genes with altered expression codes induced the proteins; this requires further investigation since the activity assay was not performed in fully denaturing conditions to detect ribonuclease activity, and the proteins can alter its mobility due to the presence of post-translational modifications. The induction of these three genes seems to respond to signals derived from developing axes, since their induction was fully abolished in decapitated seedlings obtained after epicotyl removal. This process has been used to generate rejuvenated cotyledons, in which some parameters associated with cotyledon senescence were delayed. Thus, epicotyl removal resulted in cotyledons with lower protein degradation (Ananieva et al.,2008; Lambert et al., 2016) and higher photosynthesis- related parameters (Wilhelmova et al., 2004; Ananieva et al., 2008; Jasid et al., 2009; Lambert et al., 2016) than normal grown seedlings. The developing axes have an elevated rate of respiration when rapidly growing that could lead to elevated Reactive Oxygen Species levels. In this way, either hydrogen peroxide as transportable signal or oxidative modification in the axes, can be considered in the cotyledons as signals to mobilise reserves from cotyledons to the rapidly growing axes (Verma et al., 2015). Indeed, several

antioxidant activities were much higher in axes than in cotyledons in common bean (Quiles et al., 2019). Alternatively, phytohormones derived from the developing axes can be the signals regulating this nutrient mobilisation. Indeed, it has been shown that the epicotyl removal in common bean resulted in a decrease in content of cytokinins (Wilhelmova et al., 2004). Several hormones can interact with other signalling molecules resulting in a complex crosstalk network acting coordinatively during germination (Wojtyla et al., 2016). Furthermore, the induction of the three ribonuclease genes in cotyledons during seedling development was not associated with a stress response to nutrients deficiency by the seedling since it was not affected by the presence of mineral nutrients during germination and early seedling development. Therefore, we can conclude that the induction of these genes should be predetermined by the developmental process needed for a correct seedling establishment.

PvRNS4 is the only T2 ribonuclease in common bean belonging to Class II (Diaz-Baena et al., 2020). In Arabidopsis, there is also a single gene within this class (Igic and Kohn, 2001), whereas two genes are found in rice and soybean (MacIntosh et al., 2010, Gho et al., 2020). The genes belonging to Class II in Arabidopsis (RNS2) and rice (*OsRNS2* and *OsRNS6*) are expressed in all the tissues (Hillwig et al., 2011; Floyd et al., 2017; Gho et al, 2020). Like AtRNS2, PvRNS4 is also predicted to be located in the vacuole. The function of AtRNS2 has been investigated in some detail, and it is tempting to speculate that PvRNS4 could have the same function as AtRNS2. It has been demonstrated that AtRNS2 is important to maintain RNA levels in cells, since *rns2* mutants accumulate rRNA in the cell (Hillwig et al., 2011). To exert its function, the RNS2 protein needs to be located in the vacuole (Floyd et al., 2017). The Arabidopsis *rns2* null mutants or the mutants with misallocated RNS2 present a phenotype of constitutive autophagy (Hillwig et al., 2011; Floyd et al., 2017). Autophagy is a conserved mechanism by which plants degrade the unwanted material, such as proteins, or nucleic acid and entire organelles, after targeting to the vacuole (Marshall et al., 2018). This process is involved in the recycling and remobilisation of nutrients at the whole plant level (Masclaux et al., 2017; Chen et al., 2019; Li et al., 2020) and in the responses to abiotic and biotic stresses

(Avin-Wittenberg et al., 2019; Signorelli et al., 2019). Therefore, once the RNS2 protein is imported to the vacuole, it would act maintaining the RNA turnover, and the accumulation of RNA in the mutants would indicate that in Arabidopsis this function is not fully replaced by other RNS proteins.

PvRNS1 is the gene with the expression that increased the most in cotyledons, from 1 to 10 DAI (1000-fold). The analysis of the deduced PvRNS1 protein sequence predicts an extracellular localisation (Diaz-Baena et al., 2020), and the pattern of expression during germination was different from that of *PvRNS4*, since *PvRNS1* expression was almost undetectable in cotyledons at 1 and 3 DAI. Therefore, a different function for PvRNS1 protein in common bean can be hypothesised. PvRNS1 belongs to Class I RNases, enzymes that have been proposed to be involved in abiotic stress responses such as salt stress and phosphate deficiency responses (Bariola et al., 1994; MacIntosh., 2010; Zheng et al., 2014; Diaz-Baena et al., 2020; Gho et al., 2020). The high level of expression of *PvRNS1* during the high nutrient mobilisation phase in cotyledons in the absence of external stress could mean a main role in ribonucleic acid mobilisation during seedling development. Efficient mobilisation processes in cotyledons could improve plant fitness. In common bean seedlings, the removal of cotyledons leads to a slowdown in the growth of the isolated axes (Lambert et al., 2016). In this way, the knowledge of the mechanisms involved in nutrient mobilisation at the whole-plant level during leaf senescence has been proposed as a way to improve important agronomic traits in crops species such as seed production and quality (Have et al., 2017). Most of the studies have been focused on nitrogen mobilisation with proteins as the main nitrogen source, but the role of nucleic acid could have been underestimated, since it is a non-negligible source of nitrogen at the same time that it is a primary source of phosphorous (Have et al., 2017).

In this paper we have demonstrated the induction of ribonuclease activities in cotyledons of common bean during the high nutrient mobilisation phase, and we have shown that S-like ribonuclease genes are induced during this process. *PvRNS1* is the gene most highly induced in cotyledons from 1 to 6 days after

imbibition with a relative induction of 1000 fold, and it is a good candidate to have a relevant role during the mobilisation of RNA in cotyledons and, therefore, further characterisation of the gene will be interesting in order to increase knowledge of RNA turnover. Since the induction seems to be dependent on signals derived from developing axes and independent from the presence of nutrients in the surroundings media, it will also be interesting to address this point to improve the efficient seedling establishment of important crops such as common bean.

5. SUPPLEMENTARY MATERIAL

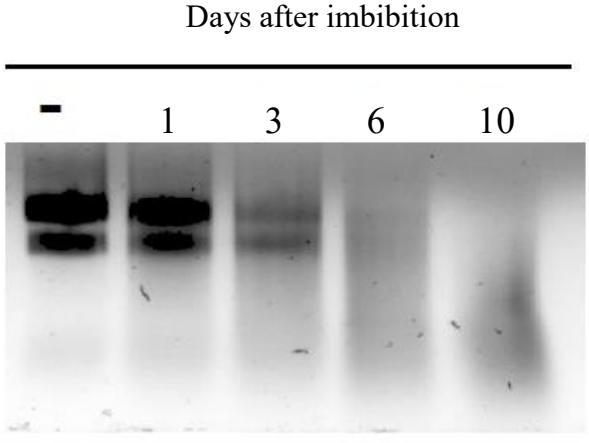

Figure S1. *Ribonuclease activity in common bean during cotyledon development Ribonuclease activity was determined as indicated in Figure 2 but normalising the activity assays per amount of fresh weight instead of amount of total protein in crude extracts The crude extracts were obtained from cotyledons at the indicated DAI and, as control, the total RNA used as substrate was incubated in the absence of crude extracts After enzymatic reactions, the remaining rRNA was visualized in agarose gels after electrophoresis*

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CHAPTER II:

NUCLEOTIDE METABOLISM IN COMMON BEAN PODS DURING SEED FILLING PHASE REVEALS THE ESSENTIAL ROLE OF SEED COATS.

Diaz-Baena, M., Delgado-Garcia, E., G.de Ravé-Prieto, I.,Galvez-Valdivieso, G., Piedras, P. Nucleotide metabolism in common bean pods during seed filling phase reveals the essential role of seed coats. (Submited to a Acta Physiologiae Plantarum . (Under review).

ABSTRACT

Common bean is a legume with high demand for human consumption and with high protein content on its seeds. The seed filling stage is a crucial step to obtain high quality seeds with a good level of nutrients. For this, it is necessary a correct communication between the different seed compartments. Nucleotides are essential components with nitrogen and phosphorous on its molecules and which metabolism in seed development has not been studied in detail. In this manuscript we have studied nucleotide metabolism in common bean pods during seed filling stage at pod valves, seed coats and embryos. Nuclease and ribonuclease activities were assayed as nucleotide-generating enzymes, and nucleotidase, nucleosidase and allantoinase as nucleotide-degrading activities. Nuclease was predominant in seed coats whereas ribonuclease was equally determined in seed coats and valves, although with differences in the three ribonucleases determined (16, 17 and 19 kDa). Nucleotidase and nucleosidase activities were detected in the three pods parts, and differently to nucleic degrading activities with significant activity in embryos. The relative expression of gene families coding for all these activities (S1 nuclease, S-like T2 ribonuclease, nucleotidase, nucleosidase and allantoinase) in the three pods parts was also studied. We have found the highest level of expression for some members of each family in seed coats. The allantoinase data suggest that nucleotide might be fully degraded in valves and seed coats but not in embryos. Overall, the data presented allow to conclude that there is an intense nucleotide metabolism in fruits during the seed filling stage with an especial involvement of seed coats in the process.

Keywords: Legumes, nuclease, ribonuclease, nucleotidase, nucleosidase, allantoinase.

1. INTRODUCTION

Common bean (*Phaseolus vulgaris*) is the most important legume used in human alimentation worldwide (Broughton et al., 2003). About 27 million tons of dried beans and 23 million green beans are produced worldwide (Gioia et al., 2019). It is an important source of vegetable proteins, therefore, could be very important as dietary protein to alleviate malnutrition or as a substitute of animal protein. Common beans are an essential food for more than 500 million people in developing countries, where they provide more than 50 % of the protein ingested by their population (Broughton et al., 2003; Graham et al., 2003). Common bean followed independent domestications processes originating two gene pools, Mesoamerican and Andean, with high diversity in each one being a unique situation among crops (Blair et al., 2012; Cortés et al., 2018). This genetically differentiated pools contain cultivar with different seed and leaf sizes, growth habits and seed coat colours and patterns.

The pods that surround the seeds have an important and dual role during seed formation as protecting the seeds as well as contributing to the nutrition of the developing seeds (Bennett et al., 2011). Seed development can be divided into three phases: the early phase in which the embryo develops all the structures, the seed filling phase characterized by rapid cell division and the maturation phase when they start to desiccate (Ali et al., 2022). During the first part of common bean seed development, the amount of reduced carbon received by the pods is larger than the needs of developing seeds, and the excess sucrose is stored in pods to be used when the sucrose supply is no longer sufficient to support the needs of the seeds (Belmont et al., 2022). The bean fruit pericarp is photosynthetically active, but the assimilation of carbon dioxide gradually decreased as the pod develops, suggesting that this process may be important for pods setting but with minor contribution to seed development (Belmont et al., 2022). Despite the importance of this process, the allocation of nutrients during seed formation is poorly understood, with most of the research being performed on nutrient mobilization from senescing leaves (Have et al., 2017).

The seed coat is entirely maternal in origin and lack a vascular system extending beyond the seed coat, which make the embryo a tissue apoplastically isolated from the mother plant (Radchuk et al., 2014). Within the seed coat, some nutrient metabolism may occur before transferring the nutrients to the apoplast (The et al., 2021). Some transiently stored compounds within the seed coat could act as nutrient buffer, and its mobilization to the embryo would promote its growth (Weber et al. 2005). Furthermore, due to this involvement, it has been proposed that early embryo growth is subjected to maternal control in legume seeds (Weber et al., 2005).

During seed filling stage, the developing seed needs to incorporate nitrogen in order to synthetize the storage proteins. The role of nitrogen mobilized from the leaves has been related to the protein content of the seed rather than the nitrogen uptake by the plant, since in most of the plant species studied the proportion of N in seed provided by remobilization from vegetative tissues is much higher (more than 70 %) than the proportion originating by N uptake post-flowering (Have et al., 2017). However, little attention has received the role of nucleic acids as nutrient supplier in this process, despite been an important reservoir of nitrogen and phosphorus. In fact, nucleic acids constitute the largest pool of organic phosphorus in plants (Veneklaas et al., 2014). Among nucleic acids, RNA is the most abundant, representing 85 % of total nucleic acids in plant cells and providing 47 % of the organic phosphorus (Veneklaas et al., 2014). Thus, nucleic acids may play a crucial role in processes involving nutrient mobilization. In wheat plants subjected to N starvation has been demonstrated that RNA catabolites contribute to nitrogen pool in this situation with purine catabolism being critical in the process (Melino et al., 2018).

The enzymes involved in the breaking down of nucleic acids are nucleases and ribonucleases, enzymes that release nucleotides. Nucleotidases are phosphatases that release the phosphate group from nucleotides yielding nucleosides, which are the substrate of nucleosidases that release the sugar moiety and the nucleobase. Nucleotide metabolism in plants has been recently revised providing deep information about how nucleotide catabolism is connected, with a special

emphasis at the cellular level (Witte and Herde, 2020). The model is raised for Arabidopsis and, therefore some variations in other plant species are possible. Purine nucleotide metabolism is particularly relevant in ureidic legumes, such as common bean, as precursor of ureides, molecules that play an important role in the transport of nitrogen in these legumes (Todd et al., 2006; Quiles et al., 2019). Purine and pyrimidine nucleotides can be synthesized de novo or through the salvage pathways, converging at the formation of nucleoside monophosphates, the substrates of phosphatases catalyzing the first step in the catabolic pathway. So far, which nucleotide phosphatases mediate dephosphorylation in vivo is an open question that need to be addressed (Witte and Herde, 2020). The next step in nucleotide catabolism is catalyzed by nucleoside hydrolases, enzymes that cleave nucleosides into ribose and nucleobases (Delgado-García et al., 2021). Nucleobase can be totally catabolized or, in the case of purine nucleobase derived as well to ureides (Witte and Herde, 2020).

During seed filling stage the pods play and essential role for seed formation, however the research carried out so far on nutrient mobilization in pods is very scarce, especially at the molecular level. In common bean, we have identified the S1 nuclease family (Lambert et al., 2016), the T2 ribonucleases members (Diaz-Baena et al., 2020). The enzymes coded by these genes could generate nucleotides from the nucleic acids. We have identified the genes belonging to the haloacid dehalogenase-like hydrolases (HAD) superfamily (Cabello-Diaz et al., 2015; Galvez-Valdivieso et al., 2020; 2021), which could code for enzymes candidate to dephosphorylate the nucleotides to nucleosides, and we have characterized two genes coding for nucleosidases (Delgado-García et al., 2021), which code for enzymes releasing base from nucleoside. We hypothesized that nucleic acids and its degradation products must contribute to provide nitrogen and phosphorous to the developing seeds. Therefore, the aim of this research has been to carry out a comprehensive study both at the level of enzymatic activity and gene expression regarding the metabolism of nucleotides during the fruit filling phase. A scheme showing the steps catalysed by the enzymes studied in this work is shown in Figure S1. A better knowledge on the seed filling phase

could be crucial to enhance either the quantity of crop yield or its nutritional properties, processes with high agronomical importance.

2. MATERIALS AND METHODS

2.1. Plant growth

Common bean (*Phaseolus vulgaris* L. Great Northern) seeds were sterilised and germinated as previously described (Diaz-Baena et al., 2021). Five days after start of imbibition, seedlings were transferred to pots containing vermiculite: perlite (3:1, *v/v*). Unless otherwise stated, the plants were grown under nitrogen fixation conditions. The seedlings were inoculated with *Rhizobium leguminosarum* bv. phaseoli strain ISP14, 5 and 12 days after imbibition and cultivated under nitrogen free media as indicated previously (Galvez-Valdivieso et al., 2013). Pods at the seed filling phase were collected 15 days after anthesis (Raso et al., 2007) from plants after 50 days' imbibition started. Pods were separated in valves and seeds, which were further separated in seed coats and embryos including enlarged cotyledons. These three parts were immediately frozen with liquid nitrogen. Frozen plant materials were pulverized to a fine powder in liquid nitrogen with mortar and stored at 80 °C until use.

To study the effect of nitrate fertilization in gene expression in seed coats, the plants were grown with medium supplemented with 10 mM nitrate (Gálvez-Valdivieso et al., 2013) and material collected as indicated above.

2.2. Preparation of crude extracts

Frozen powder was homogenised with extraction buffer (50 mM TES buffer (pH 7.0) containing 0.15 % (*w/v*) sodium deoxycholate) using 1:4, v/v ratio. After centrifugation at 15000 *g* for 10 min at 4 °C, the supernatants were considered as crude extracts.

2.3. Total soluble protein determination

The soluble protein concentration was estimated using the commercial Bio-Rad protein assay (Bio-Rad, Madrid, Spain) based on the Bradford dye-binding method (Bradford 1976) and using bovine serum albumin as the standard.

2.4. Determination of nuclease and ribonuclease activities

In vitro assays. In vitro activity assay was carried out as described in Wood et al. (1998), with some modifications. The assay reaction mixture was composed of the appropriate amount of crude extract, bovine serum albumin at a final concentration of 0.08 mg/mL, buffer 50 mM (acetate buffer pH 5.5 for ribonuclease activity or TES buffer pH 7 for nuclease activity) and as substrate RNA from torula yeast or DNA from salmon testes dissolved in milliQ water at a final concentration of 0.5 mg/mL. For ssDNA, DNA was denatured after boiling for 10 min. The final volume of the reaction mixture was 0.6 mL, and the reaction was carried out in a thermostated bath at 40 °C for 40 minutes. Aliquots of 0.2 mL were taken before and after the reaction, nucleic acids were precipitated o/n at -80 °C by adding 0.1 volume of 7.5 M ammonium acetate and 2.5 volumes of 100 % ethanol. The samples were centrifuged at 15,000 *g* at 4 °C for 15 min. The absorbance of the supernatant was measured at 260 nm. One Unit of enzymatic activity is defined as the amount of enzyme that increase 1 unit the absorbance at 260 nm.

In gel assays. In gel nucleases and ribonucleases activities were determined as described by Lambert et al. (2014) and Diaz-Baena et al. (2021), respectively.

2.5. Determination of nucleotidase activity

Enzyme activity was determined based on the appearance of phosphate present in the reaction mixture, as indicated by Cabello-Diaz et al. (2015). The standard reaction mix contained 2.5 mM of nucleotide (ADP, GMP, IMP) as substrate in 50 mM TES-HCl buffer pH 7 and an adequate amount of crude extract. One unit of enzymatic activity is defined as the amount of enzyme catalysing the production of 1 µmol of phosphate per minute.

2.6. Determination of nucleosidase activity

Nucleosidase activity was determined by HPLC following the hydrolysis of nucleosides as described in Delgado-García et al. (2021) using xanthosine 2 mM as substrate. One unit of enzymatic activity is defined as the amount of enzyme catalysing the production of 1 µmol of xanthine per minute.

2.7. Determination of allantoinase activity

Allantoinase activity was determined as previously described by Quiles et al. (2019). One unit of enzymatic activity is defined as the amount of enzyme catalysing the production of 1 µmol of allantoic acid per minute.

2.8. RNA isolation and cDNA synthesis

Total RNA was extracted from 50 mg of powdered tissue using the NZYol Reagent (NZYTECH, Lisbon, Portugal) following the manufacturer's instructions but including an additional LiCl precipitation step at the end of the procedure to improve the RNA quality. The RNA concentration was determined using a nanoVue Plus Spectrophotometer (GE Healthcare, Little Chalfont, UK). cDNA was carried out as described in Diaz-Baena et al. (2021).

2.9. Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was carried out as previously described (Diaz-Baena et al., 2021) with a CFX system (Bio-Rad) using the iTaq Universal SYBR Green Supermix (Bio Rad). The specificity of each pair of primers was verified by RT-PCR and sequencing of the products amplified and following the amplicon dissociation curves. For all the primer sets used the efficiency was higher than 90 %. The primers used in this study are given in Table S1.

2.10. Statistical Analyses.

All results are means of three independent experiments with two technical replicates. The analyses performed are indicated in the legend to figures.

3. RESULTS

3.1. Nucleic acid degrading activities in pods during filling stage and expression analysis of nucleases S1 and ribonucleases T2 S-like

Common bean pods at the seed filling stage were separated in valves and seeds, and the latter were further separated in seed coats and embryos that included cotyledons (Figure 1).

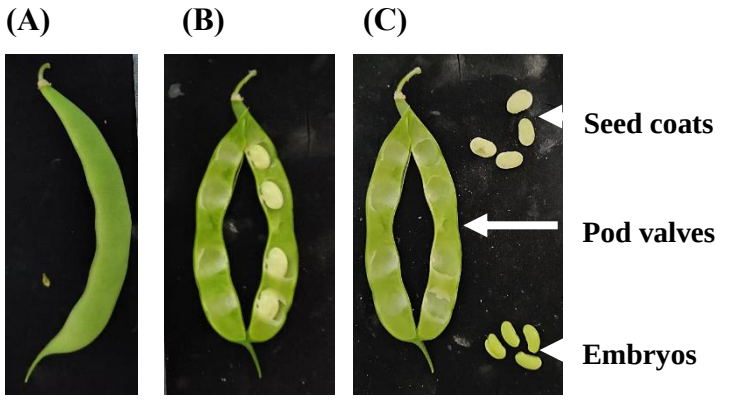

Figure 1. *Photographs of French bean pods during seed filling stage. (A) Representative whole pod. (B) Pod opened longitudinally. (C) The three tissues analysed in the present manuscript: the fruit was separated in pod valves and seeds, and the latter further separated in seed coats and embryos containing the developing cotyledons.*

Nuclease activities were assayed in the crude extracts obtained from those parts of the pods with dsDNA, ssDNA and RNA as substrates using the in vitro assay (Figure 2). With DNA as substrate, the specific activity was mainly determined in seed coats both with dsDNA (Figure 2A) and ssDNA (Figure 2B), being the activity higher with ssDNA than with dsDNA. With RNA as substrate, the specific activity was high in valves and seed coats and undetectable in embryos (Figure 2C).

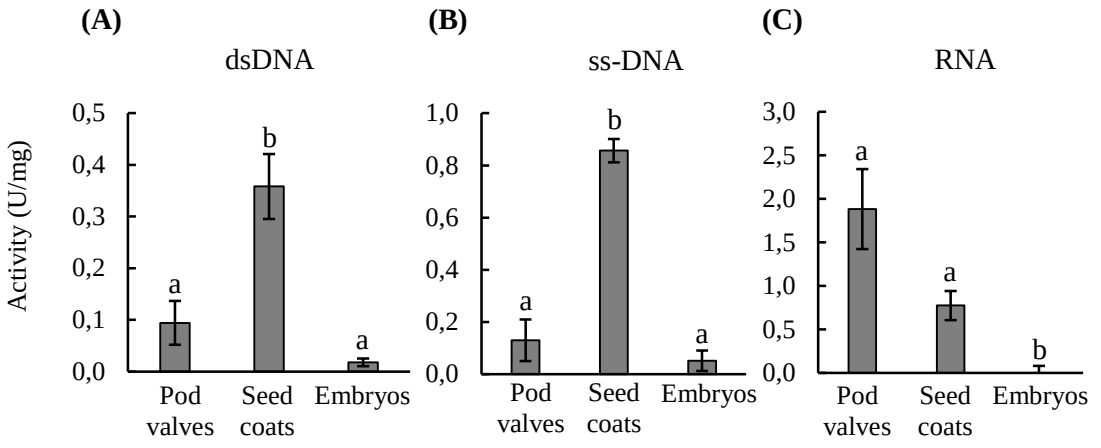

Figure 2. *In vitro nucleic acids degrading activities in common bean pods separated in valves, seed coats and embryos during seed filling stage. Nucleic acid degrading activities with dsDNA (A), ssDNA (B) or RNA (C) were determined in vitro using crude extracts obtained from each tissue. Values are means ±SE of three independent biological samples with 2 technical replicates. Different letters indicate significant differences among the tissues as analyzed by ANOVA followed by Tukey's post hoc analysis ($p \leq 0.05$).*

In order to correlate the in vitro activity with discrete protein bands, the activities were assayed as well using in-gel assays. With ssDNA, a main activity was obtained in seed coat with apparent molecular weight of 30 kDa (Figure 3A). The activity for this protein was higher at neutral than an acidic pH. With total RNA as substrate, at least four proteins with activity were determined with apparent molecular weights of 30, 19, 17 and 16 kDa (Figure 3B). The 30 kDa protein probably correspond to the same identified with ssDNA as substrate and, therefore, it was mainly detected in seed coat at neutral pH. The 19, 17 and 16 kDa proteins showed higher activity at acidic than at neutral pH (Figure 3B). In contrast to the in vitro assays, some weak signal of ribonuclease activity corresponding to the 17 and 16 kDa proteins was determined in embryos using the in-gel assay (Figure 3B).

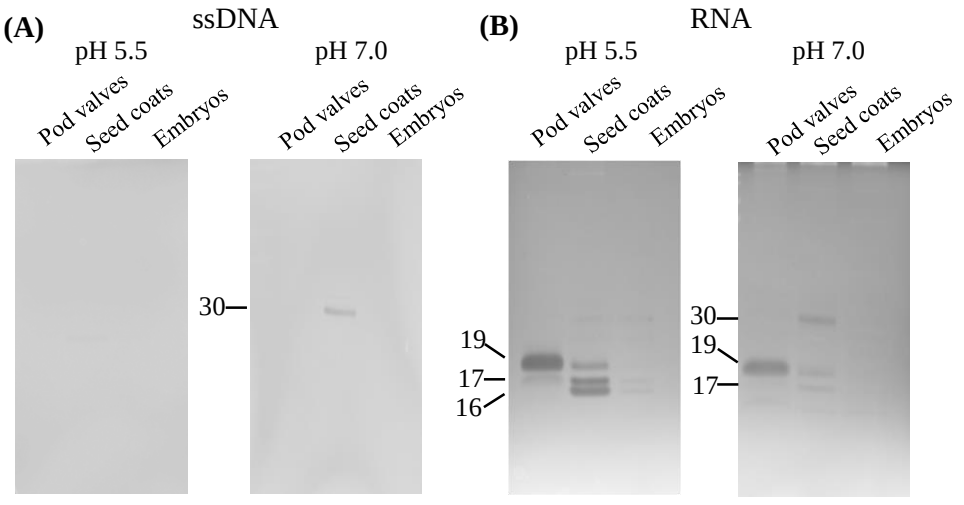

Figure 3. *In gel assays of nuclease and ribonuclease activities. In gel assays with ssDNA (A) or RNA Bb) as substrates were performed at pH 5.5 and 7.0 using crude extracts obtained from valves, seed coats and embryos from pods at the seed filling stage. The predicted molecular masses (kDa) for the protein with activity as indicated in the left of each panel.*

The expression of the genes of the S1 nuclease and S-like T2 ribonuclease families were analysed in these pods parts during the seed filling phase. Regarding the S1 nucleases, the genes *PVN3*, *PVN4* and *PVN5* were expressed in valves and seed coats, whereas in seeds the main gene expressed was *PVN3*. The genes *PVN3* and *PVN5* presented the highest expression in seed coats whereas the gene *PVN4* showed similar expression values in valves and seed coats (Figure 4A).

The expression analysis of the four S-like T2 ribonuclease genes showed that all of them were expressed both in valves and seed coats although with different relative levels of expression (Figure 4B). In valves the most expressed genes are *PvRNS1* and *PvRNS4* whereas in seed coats are *PvRNS3* and *PvRNS4*. In embryos only *PvRNS3* and *PvRNS4* were detected (Figure 4B). PvRNS1 was more expressed in valves and *PvRNS3* in seed coats than in other tissues. *PvRNS4* was equally expressed in the three tissues (Figure 4B).

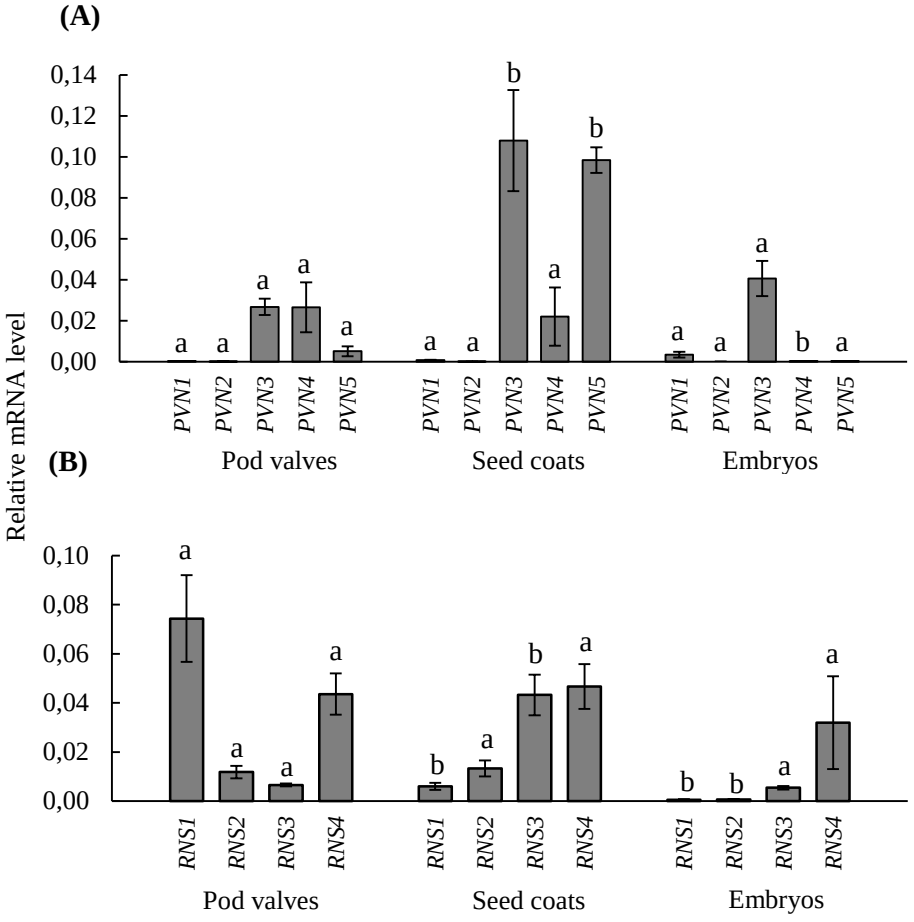

Figure 4. *Expression pattern of S1 nucleases and S-like T2 ribonucleases in pods of common bean. S1 nucleases (PVN1 to PVN5) (A) and S-like T2 ribonucleases (RNS1 to RNS4) (B) expression analysis was performed using qRT-PCR on total RNA samples extracted from valves, seed coats and embryos of common bean pods at seed filling stage. Results were normalized with the geometric mean of actin-2 and ubiquitin genes and analysed using the* $2^{-\Delta\Delta CT}$ *method. Values are mean ± SE of three independent biological samples with 2 technical replicates. Different letters indicate significant differences among tissues for the same gene as analysed by ANOVA followed by Tukey's post hoc analysis (*$p \leq 0.05$*).*

3.2. Nucleotidase activity and analysis of the expression of the genes belonging to the HAD family of putative nucleotidases

Phosphatase activity with three nucleotides as substrates was assayed in crude extracts from valves, seed coats and embryos during the seed filling stage (Figure 5). These three nucleotides were chosen based on the previous data obtained with nucleotides in common bean crude extracts (Cabello-Diaz et al., 2015; Galvez-Valdivieso et al., 2020; 2021). With the three substrates, the highest activity was obtained in valves followed by seed coat, whereas the lowest activity was observed in embryos. With ADP the activity was higher than with the nucleoside monophosphate GMP and IMP (Figure 5).

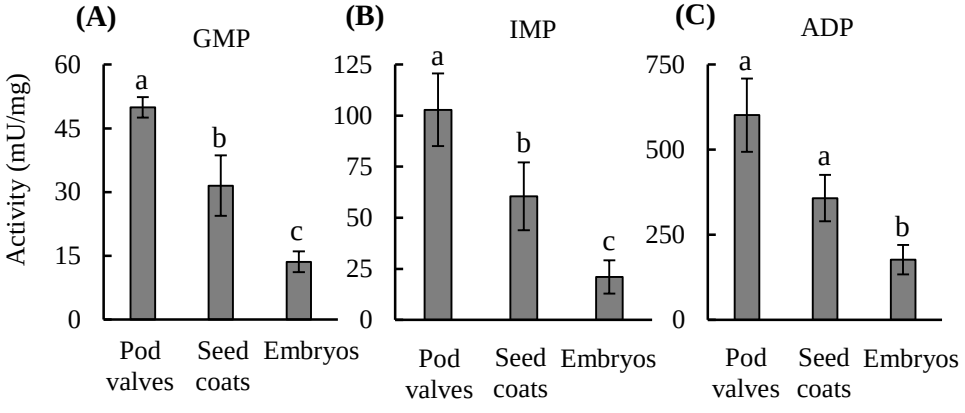

Figure 5. *Nucleotidase activity with different nucleotides. The activity was tested with GMP (A), IMP (B) and ADP (C) as substrates in crude extracts obtained from valves, seed coats and embryos of common bean pods at seed filling stage. Means ± SEs of three independent biological samples with 2 technical replicates. Different letters indicate significant differences among the tissues for the same substrate as analyzed by ANOVA followed by Tukey's post hoc analysis ($p \leq 0.05$).*

In addition, the expression of eleven genes belonging to the HAD family was analysed (Figure 6). The expression of *PvNTD10* was extremely high in valves with an expression level at least two orders of magnitude higher than the rest of genes expressed in any part of the pods (Figure 6). *PvNTD9* was expressed specifically in seeds, both in seed coats and embryos, being the gene with higher

expression in both tissues. *PvNTD1* and *PvNTD2* were expressed in the three fruits parts, *PvNTD6* in both valves and seed coats, and the expression of *PvNTD7* was detected only in seed coats (Figure 6).

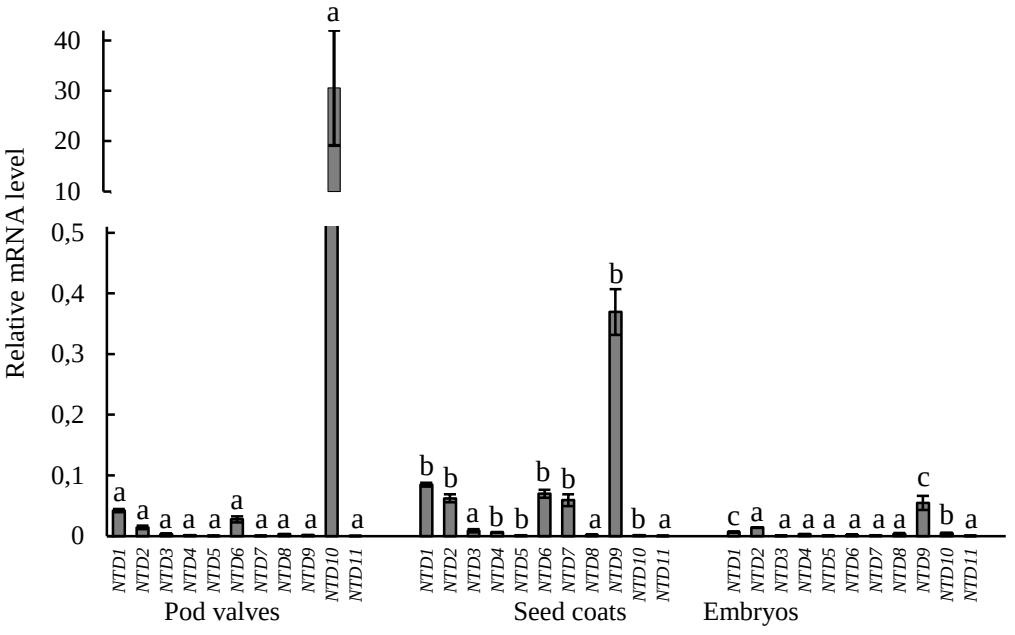

Figure 6. *Expression pattern of HAD family of putative nucleotidases (PvNTD1 to PvNTD11) in valves, seed coats and embryos of common bean pods at seed filling stage. Expression analysis was performed using qRT-PCR on total RNA samples extracted from valves, seed coats and embryos of common bean pods at seed filling stage. The relative expression level was normalised using the geometric mean of two reference genes and analysed using the* $2^{-\Delta\Delta CT}$ *method. Values are mean ±SE of 3 independent biological samples with 2 technical replicates. Different letters indicate significant differences among tissues for the same gene as analyzed by ANOVA followed by Tukey's post hoc analysis* ($p \leq 0.05$).

3.3. Nucleosidase activity and expression analysis of PvNSH1 y 2

In the same crude extracts, the nucleosidase activity was assayed with xanthosine as substrate, showing that activity was higher in valves and seed coats than in embryos (Figure 7A). This activity has some similarities with nucleotidase activity, since it was detected in all the pods parts analysed, although the nucleosidase activity in seed coats was the same that in valves (Figure 7A).

The expression of two nucleosidase genes described in common bean was analysed. *PvNSH1* and *PvNSH2* were detected in the three fruit parts analysed (Figure 7B). In seed coats, *PvNSH1* showed higher expression than *PvNSH2* being its expression the highest for both genes in any fruit part (Figure 7B). The expression of *PvNSH2* showed little differences in the three fruit parts analysed. The expression of *PvNSH2* was and similar to *PvNSH1* in valves and embryos (Figure 7B).

3.4. Nitrate fertilization effect on gene expression in seed coats.

All the results have demonstrated high nucleotide metabolism in seed coats and suggested an essential role for this tissue in seed filling. This prompted us to compare expression of the genes most highly expressed from each family analysed in seed coats from fruits obtained from plants either growth under nitrogen fixing conditions or fertilized with nitrate. We determined the gene expression for nucleases (*PVN3*, *PVN4* and *PVN5*), S-like T2 ribonucleases (*PvRNS1* to *PvRNS4*), nucleotidases genes with expression in seed coats (*PvNTD1*, *2*, *6*, *7* and *9*), nucleosidase (*PvNSH1* and *2*) and allantoinase (*ALN1*). The gene expression values obtained for all the gene analysed in seed coats were similar in both nitrogen regimes utilized (Figure S2) without statistical differences.

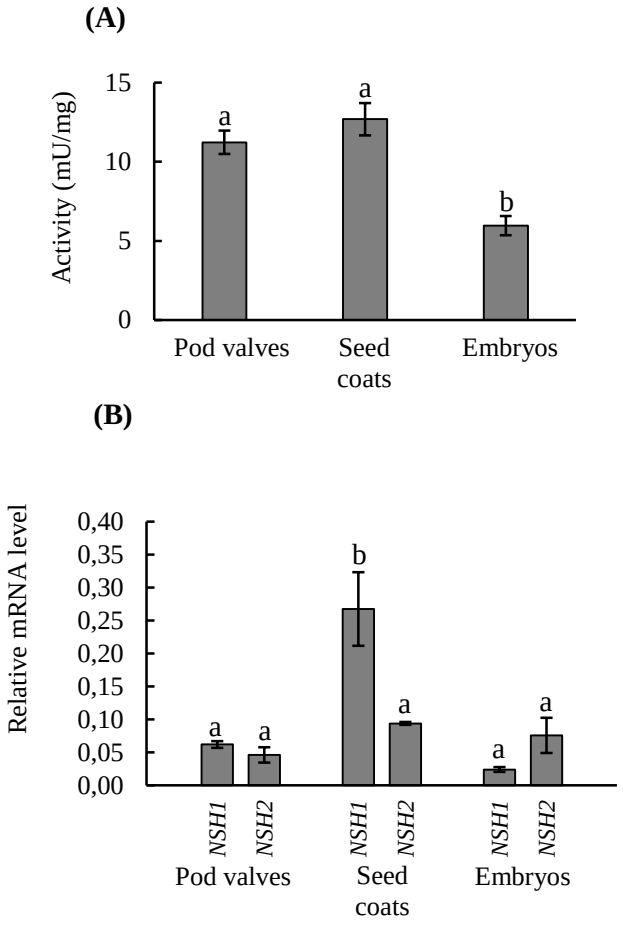

Figure 7. *Nucleosidase activity and expression pattern of two nucleosidase genes, PvNSH1 and PvNSH2, in pods of common bean. (A) Nucleosidase activity with xanthosine as substrate was determined in crude extracts obtained from valves, seed coats and embryos of common bean pods at seed filling stage. Means ± SEs of three independent biological samples with 2 technical replicates. (B) Expression analysis of PvNSH1 and PvNSH2 was performed using qRT-PCR on total RNA samples extracted from valves, seed coats and embryos of common bean pods at seed filling stage. Results were normalized with the geometric mean of actin-2 and ubiquitin genes and analysed using the* $2^{-\Delta\Delta CT}$ *method. Values are mean ± SE of three independent biological samples with 2 technical replicates. Different letters indicate significant differences among tissues for activity (a) or for relative expression for the same gene as analyzed by ANOVA followed by Tukey's post hoc analysis (*$p \leq 0.05$*).*

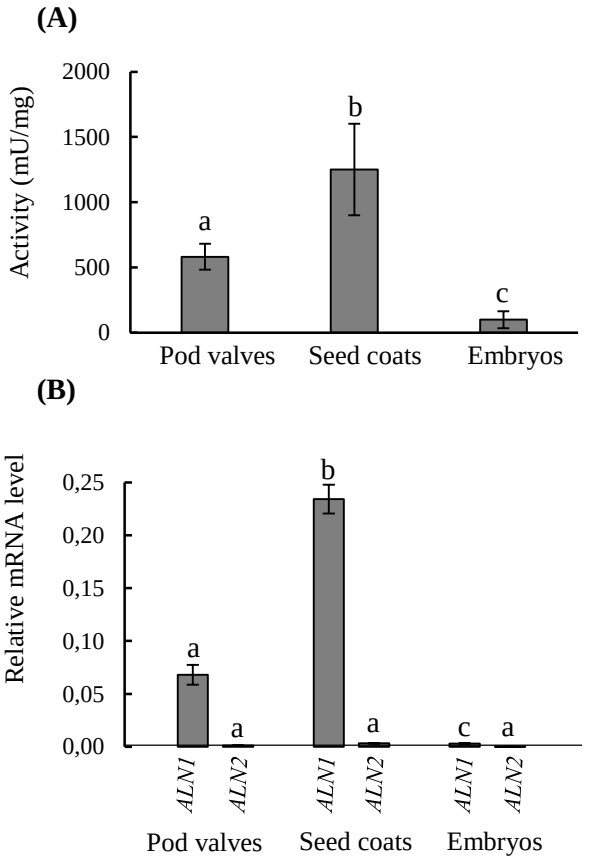

Figure 8. *Allantoinase activity and expression pattern of two allantoinase genes, PvALN1 and PvALN2, in pods of common bean. (A) Allantoinase activity was assayed in crude extracts obtained from valves, seed coats and embryos of common bean pods at the seed filling phase. Means ± SEs of three in-dependent biological samples with 2 technical replicates. (B) Expression analysis of PvALN1 and PvALN2 was performed using qRT-PCR on total RNA samples extracted from valves, seed coats and embryos of common bean pods at seed filling stage. Results were normalized with the geometric mean of actin-2 and ubiquitin genes and analysed using the* $2^{-\Delta\Delta CT}$ *method. Values are mean ±SE of three independent biological samples with 2 technical replicates. Different letters indicate significant differences among tissues for activity (a) or for relative expression for the same gene as analyzed by ANOVA followed by Tukey's post hoc analysis (*$p \leq 0.05$*).*

4. DISCUSSION

When ureidic legumes (common bean or soybean, among others) grow under nitrogen fixing conditions, they transport most of the fixed nitrogen in their nodules to the upper parts of the plants as ureides, allantoin and allantoic acid, which are synthetized from purinic nucleotides (Todd et al., 2006). The nodules are fully active during vegetative growth, and the synthesized ureides are mainly transported from nodules to leaves via the xylem. Once in the leaves, ureides can be used as nitrogen source. Although some ureides may exit the xylem to go to the phloem for direct supply of growing sinks (Pelissier and Tegeder, 2007). When the development of reproductive tissues begins, nutrients stored in the source tissues are degraded so that the released products would join the nutrients supplied by roots. During fruit development, the seeds receive nitrogen from roots and leaves, as well as from the degradation of compounds stored in valves. Ureide metabolism could have additional roles in legumes to its involvement as an efficient nitrogen transport molecule. It has been shown that altering the ureide content by the overexpression of an ureide transporter (UPS) in either fixing or non-fixing soybean plants resulted in increased nitrogen acquisition by roots and a rebalancing of nitrogen availability through the plants (Carter and Tegeder, 2016; Thu et al., 2020; Lu et al., 2022). Results obtained with UPS-overexpressing rice suggest that this role is not exclusive for legumes (Redillas et al., 2019). In addition, it has been suggested that ureides or related compounds might operate as a master regulator that responds to changes in nitrogen homeostasis and allows the adjustment of the uptake, metabolism and allocation of nitrogen (Lu et al., 2022).

Legumes seeds are rich in proteins and, therefore, the knowledge in the process of legume seed development could lead to a more sustainable source for human protein consumption. The goal of increasing seed protein content confronts to the fact that the increase in protein content is inversely related to fruit yield. Strategies such as the identification and modulation of nitrogen transporters (Joaquim et al., 2022) or the development of crops that effectively obtain, distribute, and utilize the available nitrogen could decrease this negative correlation (The et al., 2021). Although most of the attention in nitrogen use

efficiency has been addressed to the use of amino acids as nitrogen source molecules (The et al., 2021), nucleic acids could act, as well, as nitrogen and phosphorous sources for the developing seeds. We prompted to analyse the metabolism of ureides and its precursors during seeds maturation, a critical process to obtain better seeds since at this stage the seeds are supplied with nutrients from the rest of the plant.

The enzymatic activities from common bean pods related to nucleotide metabolism analysed in valves and seed coats, both maternal tissues, were high for all the activities except for nuclease in valves. Nucleic acid degrading activities in fruits has been studied only in a few cases. In banana peel, nucleases are induced during ripening and overripening treatment (Ramirez-Sanchez et al., 2018), and in ethylene treated immature cucumber fruit induction of two bifunctional nucleases and four ribonucleases were observed (Lee et al., 2015). The pattern of these activities in common bean seed coats fits to the programmed cell death model in which alkaline bifunctional nucleases initiate DNA and RNA degradation in the nucleus, and the acidic RNA specific nucleases degrade residual RNA under acidic conditions upon loss of cellular integrity (Sugiyama et al., 2000). The presence of nucleotidase, nucleosidase and allantoinase activities in both maternal tissues indicates that some of the nucleotides are fully degraded before transferring to the embryos. The seed coat lack of vascular connection with the embryo and is the tissue in which occurs the phloem unloading (Radchuk et al., 2014). The presence of nucleosidase activity in seed coats collaborates the complete degradation of nucleotides in seed coats since nucleosidase has been proposed as indication for a shift toward nucleotide catabolism rather than to salvage, since nucleobases are less effectively salvaged compared to nucleotides and nucleosides (Girke et al., 2014). The high level of enzymatic activities in maternal tissues corroborate the essential role in providing nutrients to developing seeds, and the adequate mobilization of nutrients to seeds would correspond to more efficient crops.

Nucleic acid degrading activities in embryos were very low, thus supporting the fact that this part of the seed is involved in the accumulation of storage reserves,

and that the uptake from the apoplast must be in simple compounds. Some nucleotidase and nucleosidase activity was also detected in embryos, suggesting that still some nutrients could be mobilized to this part as nucleotides or nucleosides, although the activities were lower than those determined in the maternal tissues, valves and seed coats. The almost absence of allantoinase activity in embryos indicate that this tissue cannot fully degrade the nucleotides. During seed filling, the developing cotyledons require nucleotides or compounds that could release them, since requirement for these compounds must be high during germination and seedling development to synthesize nucleic acid required for seedling development. At this early stage of germination, the salvage of bases or nucleosides is higher than the de novo synthesis, and these should come from compounds stored in cotyledons during its development (Ashihara et al., 2018). Common bean accumulates nucleic acids in cotyledons because of endoreduplication (Rewers et al., 2014), a process consisting in the replication of the nuclear genome in the absence of mitosis leading to elevated nuclear gene content and polyploidy. In fact, nucleic acid degrading activities have been detected in common bean seedlings during germination and seedling development (Lambert et al., 2016; Diaz-Baena et al., 2021), and coincidently with nucleosidase activity induction (Delgado-García et al., 2021) and ureide accumulation (Quiles et al., 2009), supporting the importance of nucleotide metabolism during seed formation and seed germination. A good supply of nucleotides for endoreduplication would be critical for improving the availability of components during germination.

Although with our analysis it is not possible to associate the enzymatic activities with the expression of some genes, we have analysed the relative expression of genes families that encode the enzymatic activities analysed to identify those that clearly could be important in the process of seed filling. It will be interesting to perform in the near future proteomic analysis in seed coat to correlate the nuclease and ribonuclease expressed genes to the corresponding enzymatic activity in gel assays.

The expression of nucleases S1 in common bean has been studied in some processes related to nutrient mobilization (Lambert et al., 2014; 2016; 2017). The analysis performed in the present study highlight a possible role of *PVN3*, *PVN4* and *PVN5* in some of the tissues of common bean fruit during the seed filling phase. Induction of *PVN4* and *PVN5* has been already reported in other nutrient mobilization processes; *PVN4* was induced during cotyledon senescence (Lambert et al., 2016) and *PVN5* in cotyledon and leaf senescence (Lambert et al., 2016; 2017). In both studies, the induction of *PVN4* and *PVN5* was coincident with the induction of alkaline nucleases (Lambert et al., 2016; 2017). Since expression of *PVN3* was not detected in those conditions (Lambert et al., 2016; 2017) and that nuclease detected in seed coats is more active at acidic pH (Figure 3), is tempting to assume that *PVN3* could encode the 30 kDa nuclease detected in seed coats at acidic conditions, although this requires further analysis.

The ribonuclease S-like T2 *PvRNS4* was detected in the three tissues of the pods analysed. Previously, we detected high level of expression of this gene in radicles and cotyledons (Diaz-Baena et al., 2020; 2021) supporting the idea that *PvRNS4* is a constitutive gene. In fact, the genes with higher similarity to *PvRNS4* in Arabidopsis and rice are also expressed in all the tissues analysed (Hillwig et al., 2011; Floyd et al., 2017; Gho et al., 2020). In addition, the function of the orthologous of *PvRNS4* in Arabidopsis (*AtRNS2*) has been investigated in some detail and it has been demonstrated that it plays an important role maintaining RNA levels in cells (Hillwig et al., 2011) and that needs to be located in the vacuole to exert its function (Floyd et al., 2017). The relative mobility of the three ribonucleases detected in seed coats in this study (16, 17 and 19 kDa) coincided with the molecular masses of ribonucleases determined in radicles (Diaz-Baena et al., 2020) and cotyledons (Diaz-Baena et al., 2021). The level of expression of *PvRNS3* in fruits tissues coincidently with the presence of acidic 16 kDa protein support the previous suggestion that this gene codes for the 16 kDa protein (Diaz-Baena et al., 2020).

Among the eleven genes bellowing to the HAD-subfamily of phosphatases previously identified in common bean (Galvez-Valdivieso et al., 2021), some of

them (*PvNTD1*, *PvNTD2*, *PvNTD6*, *PvNTD7*, *PvNTD9* and *PvNTD10*) have been detected in some tissues from pods. *PvNTD10* is expressed only in valves and exhibits a level of expression that greatly exceeds the expression of the rest of the NTD genes in any part of fruits. However, *PvNTD10* corresponds to a previously identified pod storage protein (PSP) (Zhong et al., 1997) and, therefore, its function may not be related to enzymatic catalysis but with storage. Furthermore, the only PvNTD that does not conserve an Asp in the domain I is *PvNTD10*, which contains a serine (Galvez-Valdivieso et al., 2021). The soybean vegetative storage protein also contains a serine, and the substitution of this amino acid for aspartate leads to a 20-fold increase on its phosphatase activity (Leelapon et al., 2004). All this could explain the lack of correlation between the high expression of *PvNTD10* in valves and the level of nucleotidase activity. The gene with significantly higher expression in seeds was *PvNTD9*, which according to its deduced sequence should be functional since the deduced protein contain all the amino acids in the active site (Galvez-Valdivieso et al., 2020).

PvNSH1 and *PvNSH2* were expressed in the three parts of the fruits as happened with nucleosidase activity. The expression of *PvNSH2* was similar in the three tissues whereas expression of *PvNSH1* was relatively higher in seed coats, with a level of expression of *PvNSH1* higher than *PvNSH2* as it was found recently in cotyledons during the start of nutrient mobilization (Delgado-García et al., 2021). It has been suggested that this gene could be more specific for pyrimidinic nucleosides and *PvNSH2* for purinic nucleosides (Delgado-García et al., 2021).

Allantoinase was expressed in seed coats and valves, being *PvALN1* the main expressed gene as it was previously described for other common bean tissues (Díaz-Leal et al., 2012). The allantoinase genes were not expressed in seeds, corroborating the lack of activity in this tissue and the hypothesis that nucleotides are not fully degraded in the developing seeds. The pattern of expression of *PvALN1* in fruits parts was coincident with the reported for the allantoin transporter from common bean, PvUPS1, in fruits (Pelissier et al., 2004). The high expression in seed coats reflects that high amount of allantoin is

incorporated in seed coats which is degraded in this tissue before transferring to the filial tissue.

As already mentioned, common bean is a very important crop both due to its consume and to its sustainable potential. In the current context of climate change, it will be very interesting to know how the seed filling process is affected by the changing situations, such as drought or heat. The increase in tolerance to both processes will be highly desirable in commercial varieties to maintain seed quality and crop yield. For this purpose, it will be important to select proper alleles from the high genetic diversity. The genetic variability of bean makes it a good model to study the stress tolerance mechanism, as it has already been described for heat (Cortés et al., 2022) and mainly for drought tolerance (Cortés et al., 2012; Cortés et al., 2013; Blair et al., 2016; Cortés and Blair, 2018).

In this study we have demonstrated that seed coats have both high nucleotide metabolism related enzymatic activities and elevated relative gene expression of some members of gene families involved in this metabolism. The seed coat is the tissue with high expression of the nucleases *PVN3* and *PVN5*, ribonuclease *PvRNS5*, nucleotidases *PvNTD1*, *PvNTD2*, *PvNTD6*, *PvNTD7* and *PvNTD9*, nucleosidase *PvNSH1* and allantoinase *PvALN1*. The high expression for these genes during seed filling stage corroborate the importance of the pathway in this tissue during this important physiological process. This tissue is maternal derived, and it has been proposed to determine the final seed size (Radchuk et al., 2014). The seed coats could function as a transient storage of compounds, and its degradation and mobilization to the seeds could act as buffer to promote the growth of the seeds (Weber et al., 2005), the data presented support the involvement of nucleotides in this process. It will be interesting in the future to address how the seed filling process is affected by environmental changing conditions. Among the different genes analysed, *PvNTD9* could have special relevance in seed filling process. It will be desirable to find any correlation of its involvement with metabolic changes in seeds.

5. SUPPLEMENTARY MATERIAL

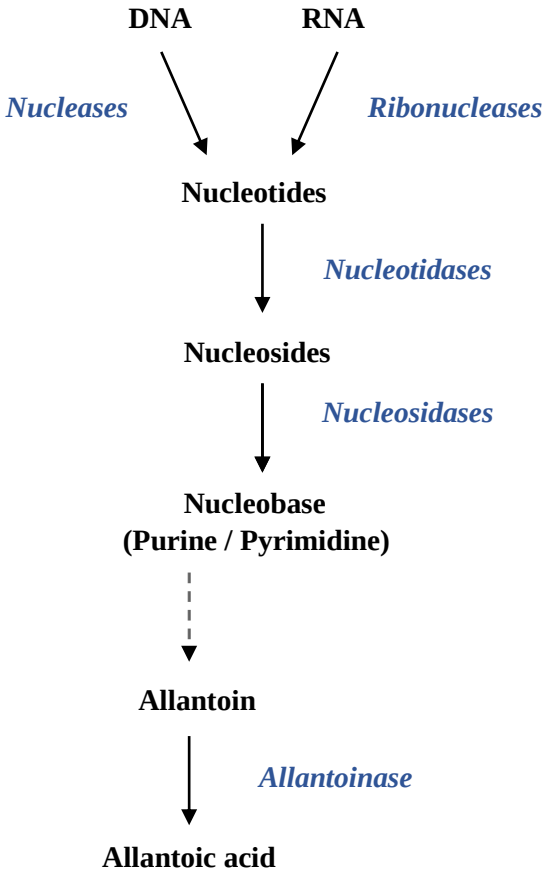

Figure S1. *Schematic overview of plant nucleotide metabolism from nucleic acids to allantoic acid.*

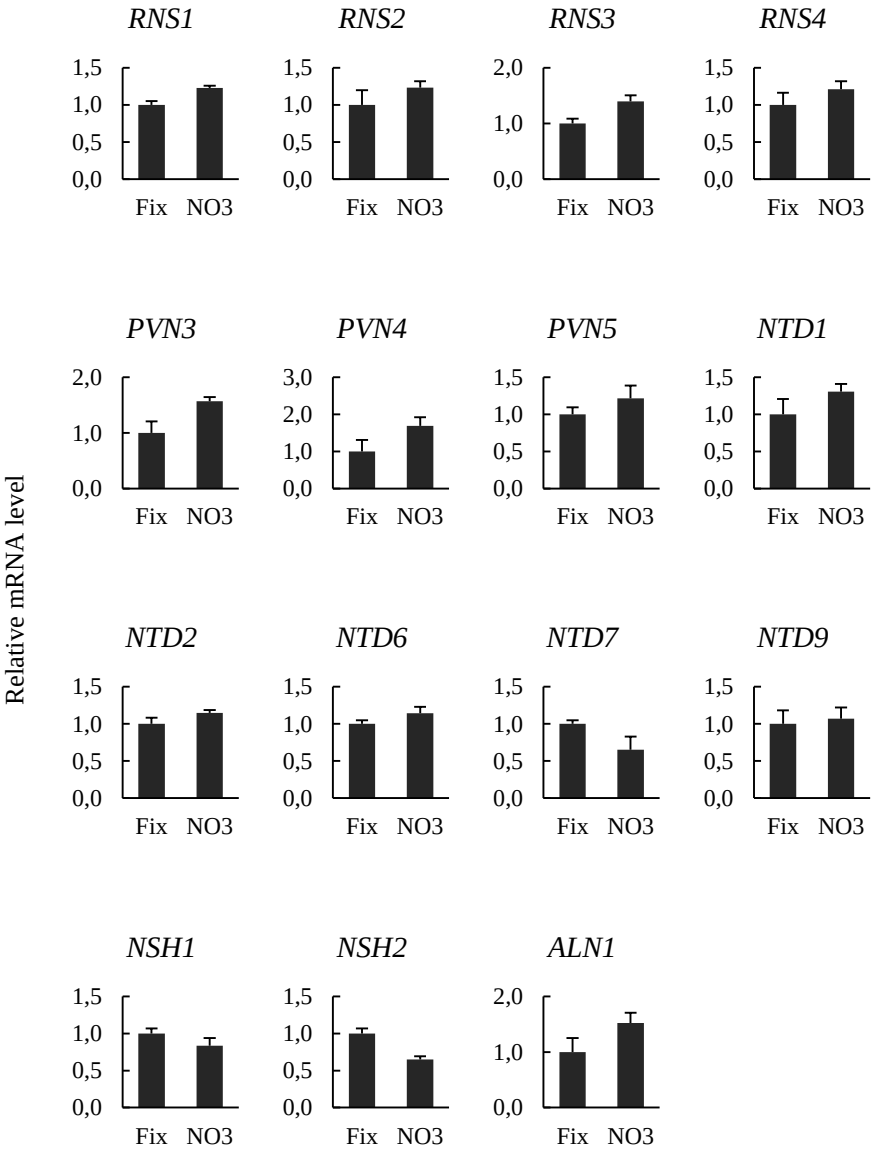

***Figure S2**. Effect of nitrate fertilization on the expression of the indicated genes in common bean seed coats from fruits. Means ± SE of three independent experiments with two replicates per experiment. For each gene, the values were normalized to the values in fixation conditions. There are not significant differences according to the Tukey test (P < 0.05).*

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CHAPTER III:

HIGH RIBONUCLEASE ACTIVITY IN TESTA OF COMMON BEAN SEEDLINGS DURING GERMINATION: IMPLICATION AND CHARACTERIZATION OF THE RIBONUCLEASE T2 PvRNS3.

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ABSTRACT

T2 ribonucleases are endoribonucleases that are found in every organism and that carry out important biological functions. In plants, T2 ribonucleases are organized into multi-gene families, and each member is thought to have a specific function. In this study, the ribonuclease activity has been analyzed in common bean seedlings during germination and it was found that the activity was very high in the testa during this process. This high activity correlated with a high level of expression of the S-like ribonuclease T2 *PvRNS3*. The protein encoded by this gene was overexpressed in *Escherichia coli* and characterized. The purified protein showed ribonuclease activity with RNA and not with DNA, confirming that *PvRNS3* encodes a ribonuclease. PvRNS3 is an acidic ribonuclease with remarkable heat stability, of which activity is inhibited by Cu and Zn, as well as by ditiotreitol (DTT). *PvRNS3* expression was also selectively induced in some stress situations, such as salt stress in radicles and wounded leaves. The high level of expression in the testa and high ribonuclease activity suggests an additional role for the testa in common bean germination, apart from being a protective barrier for embryos in seeds. The putative role of this ribonuclease in the extracellular space after seed hydration and release to the surrounding space to improve seedling fitness is discussed.

Keywords: germination; ribonuclease; testa; common bean; ribonucleic acid; wounding; stress; nutrient mobilization.

1. INTRODUCTION

Ribonucleases are very important enzymes for all organisms, as they are involved in RNA metabolism. Ribonucleases can act on single-stranded, double-stranded, or DNA- RNA hybrid substrates. The transferase-type ribonucleases are enzymes that hydrolyse single-stranded RNA, forming oligo- or mononucleotides with a terminal 3′-phosphate (Deshpande et al., 2002), and they have been classified as RNase A, RNase T1, and RNase T2 (Luhtala and Parker, 2010).

Ribonucleases belonging to the T2 family are the most widely distributed and have been described in bacteria, fungi, and eukaryotes (Luhtala and Parker, 2010). The T2 ribonucleases are endoribonucleases with little substrate specificity. They generally cleave at all four bases, and in most cases, their pH optimum is acidic (Luhtala and Parker, 2010). This family has two conserved active sites, CAS I and CAS II. Each domain contains a highly conserved His residue that is catalytically important (Irie, 1999). Mutations of these histidine residues lead to inactivation of the enzyme (Deshpande et al., 2002). The structure of human RNase T2 has four disulphide bridges (Thorn et al., 2012), which are conserved in most of the plant RNases (Diaz-Baena et al., 2020). The enzymatic reaction occurs in two steps, transphosphorylation and hydrolysis, with the conserved histidine being required in both steps (Luhtala and Parker, 2010).

At least one member of the RNase T2 family has been found in every eukaryotic genome that has been sequenced, suggesting that these enzymes have an important biological function. In humans, only one member of the T2 ribonucleases has been found (Thorn et al., 2012), and the same happened in *Drosophila melanogaster* (Ambrosio et al., 2014). However, in plants, the T2 ribonuclease family is composed of many different members, and there is evidence of frequent duplications/gene losses that resulted in different numbers of genes in different species (MacIntosh et al., 2020). For instance, the RNase T2 family is composed of five members in Arabidopsis (Igic and Kohn, 2001), eight in rice (MacIntosh et al., 2010), thirteen in common bean (Diaz-Baena et

al., 2020) and soybean (Azizkhani et al., 2021), and twenty-one in *Eucommia ulmoides* (Qing et al., 2010). This suggests that besides the ancestral function of RNases, these proteins have acquired a variety of biological functions in plants.

The plant ribonuclease T2 family can be divided into two subfamilies, called S-ribonucleases and S-like ribonucleases (Wei et al., 2006). The S-ribonucleases have been postulated to be involved in self-incompatibility, whereas the S-like ribonucleases participate in a wide range of physiological processes, such as biotic and abiotic stress responses, the maintenance of phosphate homeostasis, or production of small RNAs (MacIntosh et al., 2020). Phylogenetic analyses of these members identified three different clades defining three different classes; Classes I and II are the S-like ribonucleases, whereas the Class III corresponds to S-ribonucleases (Ramanauskas and Igic, 2017; Díaz-Baena et al., 2020; Gho et al., 2020; Qing et al., 2021).

Class I includes proteins postulated to be involved in a variety of stress responses, and its members show evidence of gene duplication, with different numbers of proteins in each species (MacIntosh et al., 2020). Induction of the expression of some Class I genes has been described in plants subjected to stress or Pi starvation (MacIntosh et al., 2020). Some of the members of this class have been hypothesized to be secreted from cells (MacIntosh et al., 2020), suggesting that these ribonucleases could be involved in the degradation of apoplastic RNA (Yoshitane and Yoshimoto, 2023). The biological significance of RNAs present outside cells needs to be reconsidered, and functions other than being cellular waste that plants could use to recycle nutrients need to be considered (Borriego and Innes, 2023). The diversity of extracellular RNAs found in the plant apoplast and their possible secretion mechanism have been recently reviewed (Borriego and Innes, 2023). In addition, recent studies also reveal that some members of the Class I T2 ribonucleases can be involved in the salvage of intracellular RNA (Yoshitane and Yoshimoto, 2023).

Class II is proposed to function in maintaining normal cellular homeostasis by recycling rRNA and typically contains a single gene per genome (MacIntosh et al., 2020). RNS2 is the Class II enzyme from Arabidopsis, which is mainly

located in the vacuole (Hillwig et al., 2011). RNS2 has been shown to be required for normal rRNA turnover (Hillwig et al., 2011; Floyd et al., 2017). The enzymatic reaction carried out by this enzyme is essential for maintaining normal cellular levels of nucleotides/nucleosides via RNA turnover (MacIntosh et al., 2020), and rns2 mutants have a deficiency in rRNA degradation leading to a decrease in cytoplasmic nucleoside and nucleotide concentrations (Kazibue et al., 2020). It has been hypothesized that this role in RNA salvage is the ancestral function of ribonuclease T2 enzymes (MacIntosh et al., 2020).

Class III proteins are not found in all plants, and these enzymes have been proposed to be mainly involved in self-incompatibility, although their involvement in other processes has also been described (MacIntosh et al., 2020).

In common bean, the S-like ribonuclease T2 subfamily is composed of four genes named *PvRNS1* to *PvRNS4* (Diaz-Baena et al., 2020). *PvRNS4* is the only T2 ribonuclease in common bean belonging to Class II, whereas the others three belong to Class I (Diaz-Baena, et al., 2020), and therefore, they are postulated to be involved in a variety of stress responses (MacIntosh et al., 2020). We have described that salt stress leads to the strong induction of *PvRNS3* expression in radicles from common bean seedlings exposed to salt stress, whereas the other three genes did not change their expression in this stress situation (Diaz-Baena et al., 2020). In relation to localization, both PvRNS3 and PvRNS1 proteins are predicted as extracellular proteins, whereas the percentages between lysosomal/vacuolar and extracellular are very similar for PvRNS2 and PvRNS4 (Diaz-Baena et al., 2020).

We are interested in unrevealing the role of nucleic acid in nutrient mobilization situations in common bean. We have recently reported the putative involvement of *PvRNS1*, *PvRNS2*, and *PvRNS4* genes in the nutrient-mobilization phase in common bean cotyledons, whereas the expression of *PvRNS3* remains unchanged in this process (Diaz-Baena et al., 2021). In this study, we performed an analysis of nucleic-acid-degrading activities in all parts of common bean seedlings during germination. The high ribonuclease activity in the testa, together with the high level of expression of *PvRNS3* led us to select this gene

for further characterization at both molecular and enzymatic levels. Purified T2 ribonucleases available in the literature are very reduced, so the characterization of heterologous expressed proteins could provide some crucial information about the properties of the enzyme. In this study, PvRNS3 was overexpressed in E. coli, and the recombinant protein was characterized. In addition, we also analysed its expression in different tissues and physiological conditions in the common bean.

2. MATERIALS AND METHODS

2.1. Plant Material and Growth Conditions

Common bean (*Phaseolus vulgaris L.* Great Northern) seeds were sterilized and germinated as indicated previously (Diaz-Baena et al., 2020). Unless otherwise stated, distilled water was added routinely to the dishes to maintain humidity.

Five days after the start of imbibition (DAI), seedlings were transferred to pots containing vermiculite: perlite (3:1, *v/v*). Unless otherwise stated, plants were cultured in media supplemented with 10 mM nitrate, as previously described (Lambert et al., 2017).

The plant materials to analyse the expression in different tissues were obtained from seedlings at 3 DAI (radicles, hypocotyls, epicotyls, cotyledons, testa) and from adult plants at 28 DAI (developing and mature leaves) and 52 DAI (developing fruits and fruits in the filling phase).

The analysis of expression in common bean radicles subjected to different treatments was performed in radicles isolated from seedlings grown in petri dishes. In all the cases, radicles from seedlings at 6 DAI were used. In the treatment with 50 mM NaCl, the seedlings were irrigated with water containing 50 mM NaCl from the beginning of imbibition. In all other cases, seedlings at 5 DAI were treated with the compounds and concentrations indicated (NaCl 200 mM, methyl jasmonate 250 µM, gibberellic acid 50 µM, abscisic acid 50 µM, salicylic acid 200 µM, 1-aminocyclopropane-1-carboxylic acid (ACC)

10 µM). The heat and cold stress treatments were caused by placing the seedlings at 40 °C and 4 °C, respectively, for 24 h.

The analysis of expression in leaves was performed in first trifoliate leaves with the following treatments. In all cases, the treatment was performed on leaves from plants at 21 DAI. Wounded leaves were obtained, damaging the leaves with forceps, and collected 24 h after the damage (22 DAI). Salt-stressed leaves were obtained from 28 DAI plants irrigated with medium supplemented with 50 mM NaCl from the time of sowing. Senescing leaves were obtained form 27 DAI plants after 6 days subjected to dark-induced senescence, as previously described (Lambert et al., 2017).

All plant material was wrapped in aluminium foil and immediately frozen in liquid nitrogen. Frozen plant materials were ground to a fine powder with mortar and pestle in liquid nitrogen and stored at −80 °C.

2.2. Crude Extracts Obtention from Plant Material

Crude extracts were generated from frozen powder via homogenization with extraction buffer (50 mM TES buffer (pH 7.0) containing 0.15 % (*w/v*) sodium deoxycholate). Approximately 100 mg of powder was transferred to a tube, buffer at a ratio of 4:1 (*v/w*) was added, and the homogenate was obtained with the use of a plastic swab. The homogenate was centrifuged at 15000 *g* for 10 min at 4 °C, and the supernatants were collected in new tubes and considered crude extracts.

2.3. Determination of Total Soluble Protein

The total soluble protein concentration was calculated according to the Bradford method (Bradford, 1976) with bovine serum albumin as standard and the commercial Bio-Rad system.

2.4. Determination of Enzymatic Activities

For polyacrylamide-gel assays, nucleic-acid-degrading activity was determined in polyacrylamide gels containing either RNA, ssDNA, or dsDNA, as described previously by Lambert et al. (2017).

For agarose gels assays, total RNA from common bean radicles was used as the substrate. Total RNA (2 µg) was incubated in a total volume of 20 µL with purified protein in 50 mM acetate (pH 5.5) at 37 °C. At times 0 and 10 min, aliquots of 10 µL were taken from the reaction mixture, and 0.33 µL of MOPS buffer pH 7 (200 mM MOPS, 50 mM sodium acetate, 1 mM EDTA-Na2) and 0.6 µL of formaldehyde were added and then gently mixed. Afterward, the mixtures were loaded in a denaturing formaldehyde agarose gel, and the reaction products were size-fractionated via electrophoresis.

For in vitro assays, a reaction mix for in vitro activity contained 125 µL of RNA or DNA (RNA from torula yeast, DNA from salmon testes, and tRNA from Saccharomyces cerevisiae) 2.4 mg/mL as the substrate, 50 µL of BSA at 1 mg/mL, crude extract or purified enzyme, and 50 mM acetate buffer pH 5.5 up to 600 µL, unless otherwise stated. The reaction was carried out at 40 °C during 30 min; 0.2 mL aliquots were extracted before and after the reaction, and then, 20 µL of ammonium acetate 7.5 M and 50 µL of ethanol were added to each tube. Nucleic acids were precipitated at −80 °C overnight. Samples were centrifuged for 15 min at 4 °C, and the absorbance at 260 nm was determined in the supernatant. One unit of enzymatic activity was defined as the amount of enzyme that catalysed an increase of 1 unit of absorbance at 260 nm per minute.

2.5. RNA Isolation and cDNA Synthesis

Total RNA was isolated from frozen plant material as indicated previously (Diaz-Baena et al., 2021). The cDNA was prepared from total RNA as previously described (Diaz-Baena et al., 2021).

2.6. Quantitative Real-Time PCR

Relative gene expression was determined via quantitative real-time PCR, as indicated previously (Diaz-Baena et al. 2020), with a CFX system (Bio-Rad, Madrid, Spain) using the iTaq Universal SYBR Green Supermix (Bio-Rad, Madrid, Spain). The primers used for the four T2 ribonucleases and the two genes used to normalize the expression (actin-2 and ubiquitin) are indicated in Table S1. The specificity of each pair of primers was verified via RT-PCR and sequencing of the products amplified and following the amplicon dissociation

curves. For all the primer sets used, the efficiency was higher than 90 %. Results were normalized to the geometric mean of the two reference genes, and relative expression was calculated from $2^{-\Delta\Delta CT}$ values (Livak and Schmittgen, 2001). Quantification was performed using at least three biological and three technical replicates for each determination.

2.7. Cloning of PvRNS3 in pET30b (+) Expression Vector

The coding region of PvRNS3 cDNA without the putative signal peptide was amplified via PCR from cDNA obtained from common bean radicles from seedlings at 5 days after the start of imbibition and subjected to 200 mM NaCl for 24 h. The primers used contained restriction sites for *SalI* and *XhoI* (Table S1). The PCR program consisted of 2 min at 95 °C, followed by 35 cycles of 95 °C at 30 s, 55 °C at 30 s, 72 °C at 50 s, and a final extension at 72 °C during 6 min. The PCR product was purified using a Nzytech commercial kit and ligated into the pSparkII vector, and the ligation product was used to transform E. coli DH5αF' cells. The insert was digested with *SalI* and *XhoI*, fractionated via electrophoresis and purified using the Nzytech commercial kit. The fragment was ligated into pET30b (+), and the plasmid was transformed into competent cells of *E. coli* BL21 (DE3) (Novagen, Merck, Madrid, Spain).

2.8. Expression and Purification of PvRNS3

The transformed cells were cultured overnight in a shaker in 4 mL of LB medium supplemented with 50 mg/L kanamycin at 37 °C. This overnight-grown culture was added to 200 mL and cultured as above until the optical density of the suspension at 600 nm reached 0.6. At that moment, isopropyl-β-d-thiogalactopyranoside (IPTG) was added to attain a final concentration of 1 mM, and the culture was incubated at 18 °C for 6 h to induce the expression of the heterologous protein. After incubation, the cells were harvested via centrifugation at 2500× g, at 4 °C for 10 min, and the pellet was stored at −80 °C until purification of the recombinant protein.

To purify the overexpressed protein, the frozen pellet was resuspended in 4 mL of lysis buffer (20 mM Na_2HPO_4, 500 mM NaCl, 10 mM imidazole, DNAse 2 mg/mL, RNAse 1 mg/mL, PMSF 0.5 mM pH 7.4). Afterwards, the resulting

homogenate was sonicated to lyse the cells using a Vibra Cell (Sonics and Materials Inc., Newton, MA, USA) with 5 pulses of 90 W and 5 s each, keeping the samples on ice during the process. After sonication, the homogenate was centrifuged at 4000 *g* for 10 min at 4 °C, and the resulting supernatant was considered the crude extract.

The crude extract was transferred to a Nickel Chelating Sepharose column (1.5 mL, GE Healthcare, Uppsala, Sweden) equilibrated with washing buffer (20 mM Na_2HPO_4, 500 mM NaCl, 10 mM Imidazole, pH 7.4). The unbound proteins were collected and named as flowthrough. Afterwards, the column was washed with 5 column volumes of washing buffer, thus collecting the fraction that was called washing. Finally, protein was eluted with 5 column volumes of elution buffer (20 mM Na_2HPO_4, 500 mM NaCl, 300 mM imidazol, pH 7.4), and this fraction was collected as eluted proteins. All these fractions, including the purified protein, were kept at 4 °C until their use.

2.9. Statistical Analysis

All results are means of at least three biological and two technical replicates. Values are mean ± SE. The analyses performed are indicated in the legend to figures. Statistical analyses were performed with SPSS Statistics, version 28.

3. RESULTS

3.1. Ribonuclease Activity and T2 S-like Gene Expression in Testa from Common Bean Seedlings

Ribonucleic acid metabolism was analysed in common bean seedlings during the germination process. To do that, seedlings at 1 and 3 days after the start of imbibition (DAI), corresponding to non-germinated and germinated seedlings, respectively, were analysed. Ribonuclease activity was very high in the testa isolated from seedlings at both 1 and 3 DAI compared to the activities from other parts of the seedlings, with the specific activity values very similar between 1 and 3 DAI (Figure 1). This activity was higher than the values obtained for embryonic axes or cotyledons (Figure 1).

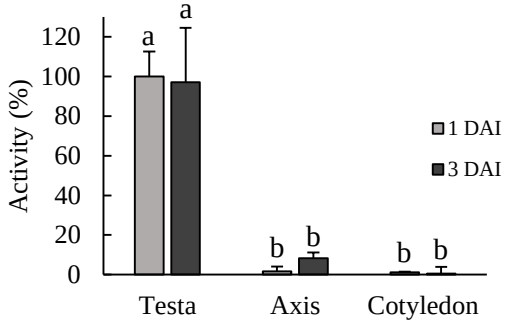

Figure 1. *In vitro ribonuclease activity in the testa, embryonic axis, and cotyledons from seedlings at 1 and 3 days after start of imbibition. The specific ribonuclease activity was normalized to 100 % according to the specific activity obtained for the testa at 1 DAI. The activities are the mean ± SE of three independent biological replicates with two technical determinations. Significant differences, according to Tukey's test, are indicated with different letters ($p \leq 0.05$).*

The high ribonuclease activity in the testa prompted us to analyze nucleic acid-degrading activities in this tissue, using ssDNA, dsDNA, and RNA as substrates. In order to differentiate acid and neutral degrading activities, the in vitro assays were performed at pH 5.5 and 7 (Figure 2). Nucleic-acid-degrading activity was observed both with RNA and ssDNA as substrates, being at both pH, the activity

was higher with RNA than with ssDNA. With dsDNA, no activity was determined at both pH values (Figure 2). A similar pattern and values of activity were obtained with the testa obtained from seedlings at 1 day after the start of imbibition.

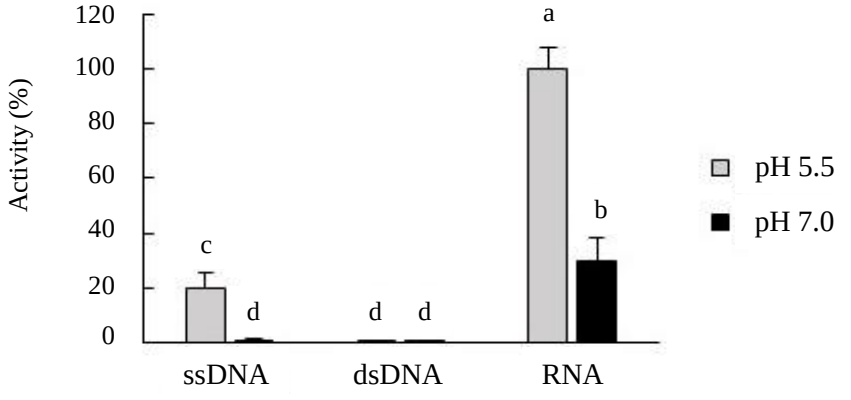

Figure 2. *In vitro nucleic acid-degrading activity with ssDNA, dsDNA, or RNA as substrates in crude extracts obtained from the testa at 3 DAI. The activity was determined both at pH 5.5 and 7.0. The specific activity was normalized to 100 % according to the specific activity obtained for RNA and pH 5.5. The activities are the mean ± SE of three independent determinations. Significant differences, according to Tukey's test, are indicated with different letters ($p \leq 0.05$).*

Once high ribonuclease activity was determined in the testa of bean seedlings, we proceeded to characterize this activity in crude extracts. High ribonuclease activity was determined at a wide pH range, between 4.5 and 6.5, with maximal activity determined at pH 5.0 (Figure 3A). Using an in-gel ribonuclease assay, three major RNA-degrading activities were detected in the testa at optimal acidic conditions, with molecular weights corresponding to 16, 17, and 19 kDa (Figure 3B) and with the major activity corresponding to the protein with an apparent molecular mass of 16 kDa (Figure 3B). However, these values cannot be related to the real molecular weights of the ribonucleases since this assay requires electrophoresis to be performed under non fully denaturing conditions. The same three proteins were determined when the enzymatic activity was assayed at pH

7.0, with the 16 kDa proteins being the most active at pH 7.0 as well. The ribonuclease activity in crude extracts from the testa was assayed in the presence of several cations in the reaction mixture, and it was found that the activity was completely inhibited by the presence of the cations Cu or Zn in the reaction mixture (Figure 3C).

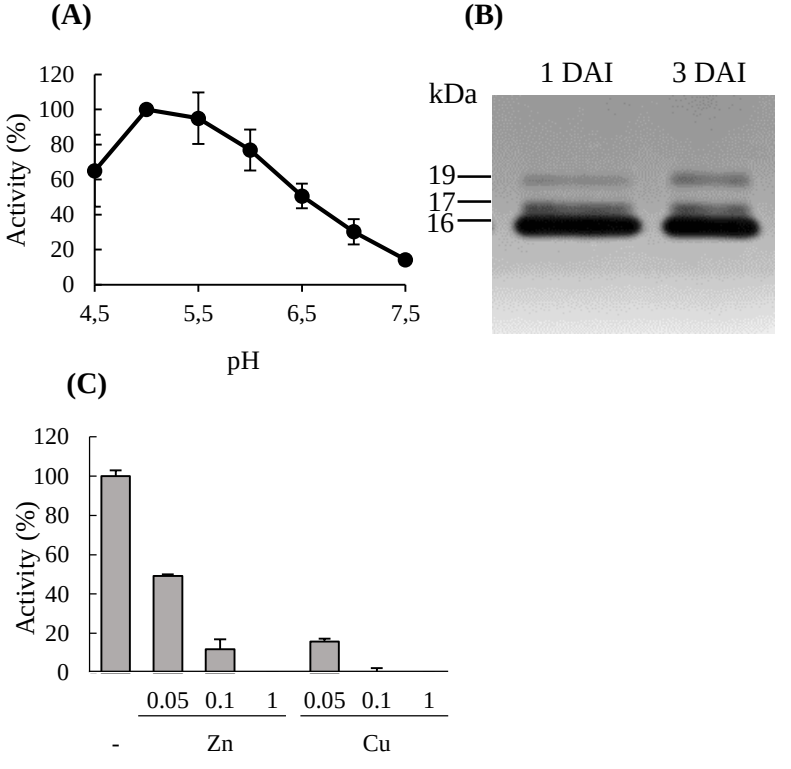

Figure 3. *(A) The activity in crude extracts from the testa obtained from seedlings at 3 days after imbibition was determined at the pH indicated. The activity was normalized to 100 % according to the highest value. The activities are the mean ± SE of three independent determinations. (B) In-gel ribonuclease activity in crude extracts from the testa obtained from seedlings at 1 and 3 days after the start of imbibition. The apparent molecular mass for each activity is indicated in the left of the panel. (C) Effect of Zn and Cu on ribonuclease activity from the testa obtained from seedlings at 3 DAI. The activity was determined with crude extracts in the presence of the indicated amounts of Zn and Cu (mM). The activities are the mean ± SE of three independent determinations. Activities were normalized to 100 % corresponding to the activity in the absence of a cation. Significant differences, according to Tukey's test, are indicated with different letters ($p \leq 0.05$).*

The high ribonuclease activity determined in the testa both at 1 and 3 DAI, prompted us to analyze the expression of the four members of the S-like T2 ribonucleases in the testa from seedlings at 1 and 3 DAI (Figure 4). High expression was observed for PvRNS3 and PvRNS4 in the testa from seedlings at both 1 and 3 DAI. Both genes were expressed with a similar level of expression (Figure 4). The expression of *PvRNS1* and *PvRNS2* was lower and did not change significantly (Figure 4).

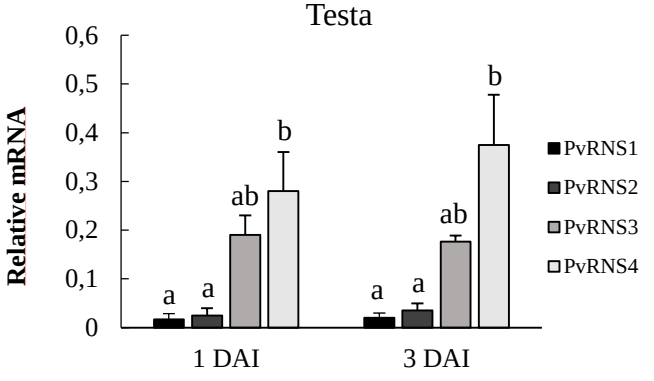

***Figure 4**. Expression pattern of S-like T2 ribonucleases in the testa from common bean seedlings at 1 and 3 days after imbibition. The relative expression level was normalized using the geometric mean of the two reference genes (actin-2 and ubiquitin), and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schimittgen, 2001). Values are the mean ± SE of three biological replicates with three technical replicates. Significant differences, according to Tukey's test, are indicated with different letters ($p \leq 0.05$).*

3.2. Sequence Analysis of PvRNS3

After the expression data obtained for the testa, the gene *PvRNS3* was chosen for further characterization since *PvRNS4* belongs to Class II and therefore is predicted to be a constitutive gene (Diaz-Baena et al., 2020). The ORF of the *PvRNS3* gene of the common bean was amplified via RT-PCR using RNA isolated from salt-stressed radicles as a template. The deduced amino acid sequence corresponds to the gene previously identified in the Phytozome database (Phvul.002G084600) (Figure S1A). The ORF encodes a deduced

protein of 227 amino acids, with a predicted signal peptide of 24 amino acids, which after processing, will yield a mature protein of 24.9 kDa (Figure S1B). The protein has an isoelectric point of 4.47 and is negatively charged at neutral pH. The protein contains 21 negatively charged residues (Asp and Glu) and 12 positively charged residues (Arg and Lys) (Figure S1B).

To investigate the structural similarity of *PvRNS3* with other S-like ribonuclease T2 genes, we analyzed the exon/intron structure of the S-like ribonuclease T2 genes in both *P. vulgaris* and *Arabidopsis*. Identical exon/intron patterns were observed among all the genes belonging to either Class I or II. All the genes classified into Class I (*PvRNS1*, *PvRNS2*, *PvRNS3*, *AtRNS1*, *AtRNS3*, *AtRNS4*, and *AtRNS5*) have four exons, whereas the two genes belonging to Class II (*PvRNS4* and *AtRNS2*) have nine exons (Figure S2). The three Class I genes in *P. vulgaris* have identical lengths for exons 2, 3, and 4 with 156, 199, and 224 bp, respectively (Figure S2). Furthermore, the three genes are in the same chromosome with *PvRNS1* and *PvRNS3* located in tandem. The conservation of the gene structure in each class strongly suggests that members of each class are evolutionarily related.

3.3. Heterologous Expression and Purification of Recombinant PvRNS3

The coding region of *PvRNS3* without the predicted signal peptide was amplified via PCR, cloned into the pET30b (+) vector, and transformed into *Escherichia coli* BL21 (DE3) cells. *E. coli* cells containing pET30-RNS3 expressed the recombinant protein in the soluble fraction after induction with IPTG, with a maximal yield when the induction was performed at 18 °C for 6 h. The recombinant protein was purified via affinity chromatography with Ni sepharose (Figure 5).

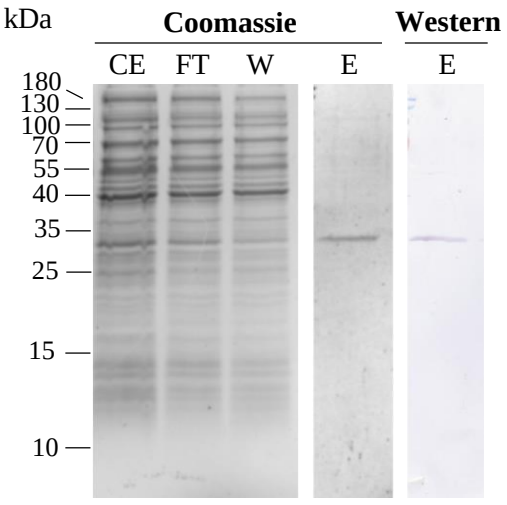

Figure 5. *Purification of recombinant PvRNS3 overexpressed in E. coli. The purification procedure is described in the Materials and Methods section. The following samples were loaded in each lane of the polyacrylamide gel: crude extract (CE), proteins not bounded to the column and collected as the flowthrough (FT), proteins bound to the column and eluted with 10 mM imidazole (W), proteins bound to the column and eluted with 300 mM imidazole (E). After SDS-PAGE, the gels were stained with Coomassie blue or analysed via Western blotting. The relative mobility and molecular weight of the standards are indicated in the left of the figure.*

The purified protein showed activity with total RNA from the torula using in-gel assays, whereas no activity was obtained with ssDNA and dsDNA from salmon sperm as substrates (Figure 6A), confirming that the purified protein is a ribonuclease. The same substrate specificity was obtained when the activity was assayed using in vitro assays. The purified PvRNS3 also showed ribonuclease activity with rRNA from the common bean (Figure 6B) and with tRNA from *Saccharomyces* (Figure 6C) as substrates.

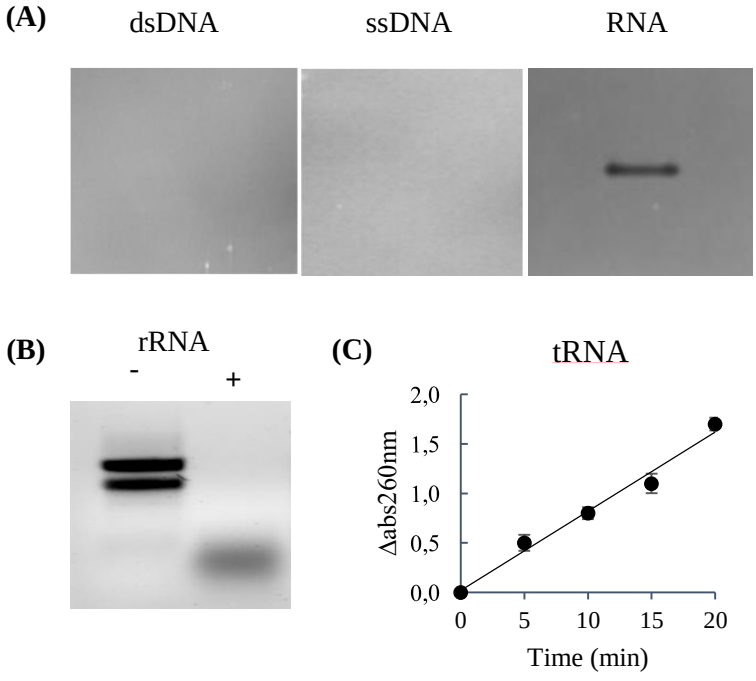

Figure 6. *Substrate specificity of purified PvRNS3. (A) Nuclease and ribonuclease activities assayed with double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), or total RNA (RNA) using the in-gel assay. (B) Ribonuclease activity assayed with rRNA using the agarose gel assay. Lane −: reaction mixture without PvRNS3 showing rRNA not degraded after incubation. Lane +: reaction mixture with purified PvRNS3, showing the degradation of rRNA. (C) Ribonuclease activity assay with tRNA as the substrate using an in vitro assay. The absorbance corresponding to tRNA degradation is represented at the indicated incubation time.*

3.4. Characterization of PvRNS3

The purified PvRNS3 showed maximal activity at temperatures in the range of 40-50 °C (Figure 7A) and at a pH range of 4.5 to 6.0 (Figure 7B). From these pH values, the activity dropped very fast, so that the purified enzyme showed not activity at neutral pH (Figure 3B). The purified protein showed a marked stability with heat denaturation, retaining all activity after 10 min at 80 °C (Figure 7C).

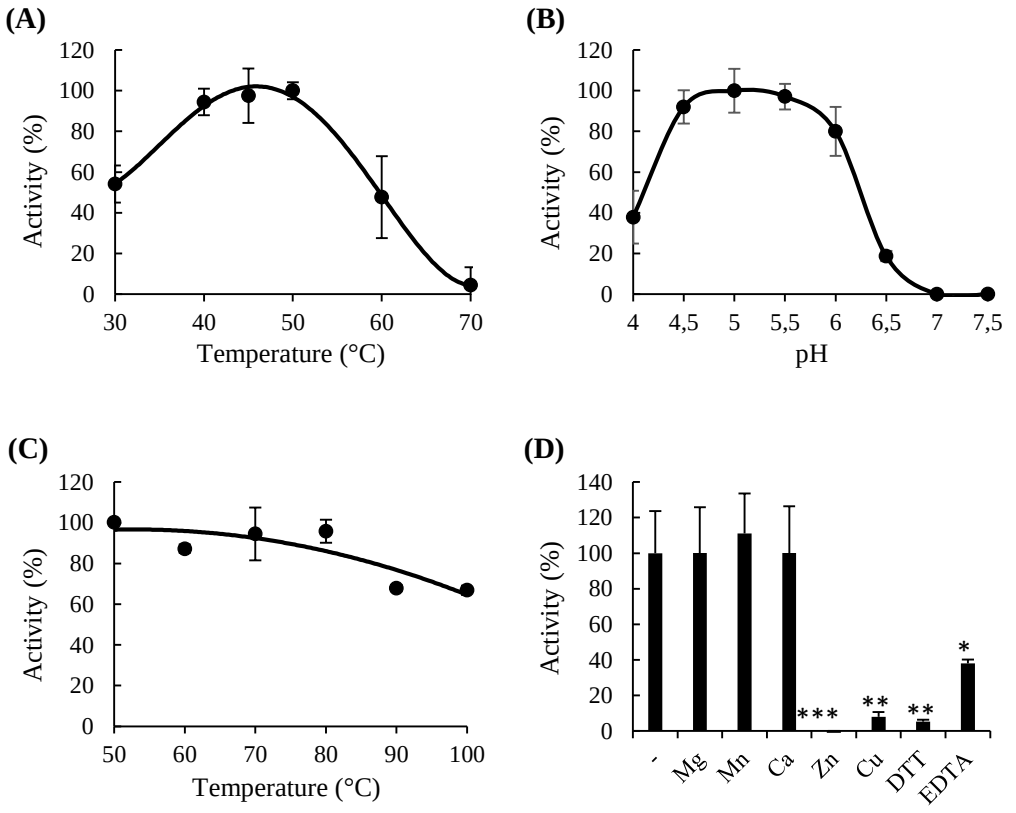

Figure 7. *Characterization of ribonuclease activity from purified PvRNS3. In all the cases, the activity was assayed with RNA from the torula using the in vitro activity assay. (A) To establish the optimum temperature, the activity was assayed at the indicated temperature for 30 min. (B) To stablish the optimum pH, the activity was assayed at the indicated pH for 30 min. (C) The thermal stability was determined by incubating it at the indicated temperature for 10 min followed by incubation at 4 °C for 15 min. The remaining activity after the treatment was assayed at 50 °C for 30 min. (D) In vitro ribonuclease activity was assayed with purified PvRNS3 in the presence of the indicated cations, DTT, or EDTA. All compounds were used at a final concentration of 2 mM, except for Cu and Zn, which were used at 0.1 mM. In all cases, the activity was normalized to 100 % according to the highest value. The activity represents the mean ± SE of three independent determinations. (*), (**) and (***) indicate a p-value lower than 0.05; 0.01 and 0.001 respectively.*

The effect of several cations and compounds on ribonuclease activity was also determined (Figure 7D). The purified protein lost almost all its activity after treatment with the reducing agent ditiotreitol (DTT) and the metal chelator

EDTA (Figure 7D). Among the cations assayed, the inclusion of Ca, Mg, or Mn ions at concentrations up to 2 mM did not affect the activity, whereas the inclusion of Zn and Cu ions at 0.1 mM completely inhibited the activity (Figure 7D).

3.5. Molecular Characterization of *PvRNS3*

The expression level of *PvRNS3* was analysed via qRT-PCR in different tissues of common bean seedlings and adult plants (Figure 8A). The *PvRNS3* transcript was particularly high in the testa isolated from developing seedlings (Figure 8A).

We analysed the effect of 24 h treatment with several hormones or stress conditions in radicles from 6-day-old seedlings (Figure 8B). None of the hormone treatments analysed (MeJA, GA, ABA, ACC, or SA) altered the expression of *PvRNS3* (Figure 8B). Similarly, neither, heat nor cold treatment altered the expression of *PvRNS3* in radicles (Figure 8B). The only treatment that induced the expression was that consisting of a salt concentration (200 mM) (Figure 8B), as it was previously identified (Diaz-Baena et al., 2020), and that was included in the analysis as the positive control.

We also wanted to analyse the expression of *PvRNS3* in the leaves of adult common bean plants exposed to several adverse situations, such as continuous dark to induce leaf senescence, salt stress, or wounding. As shown in Figure 8C, *PvRNS3* expression remained unchanged after induced senescence or in plants grown under salt stress situations but was strongly induced in wounded leaves.

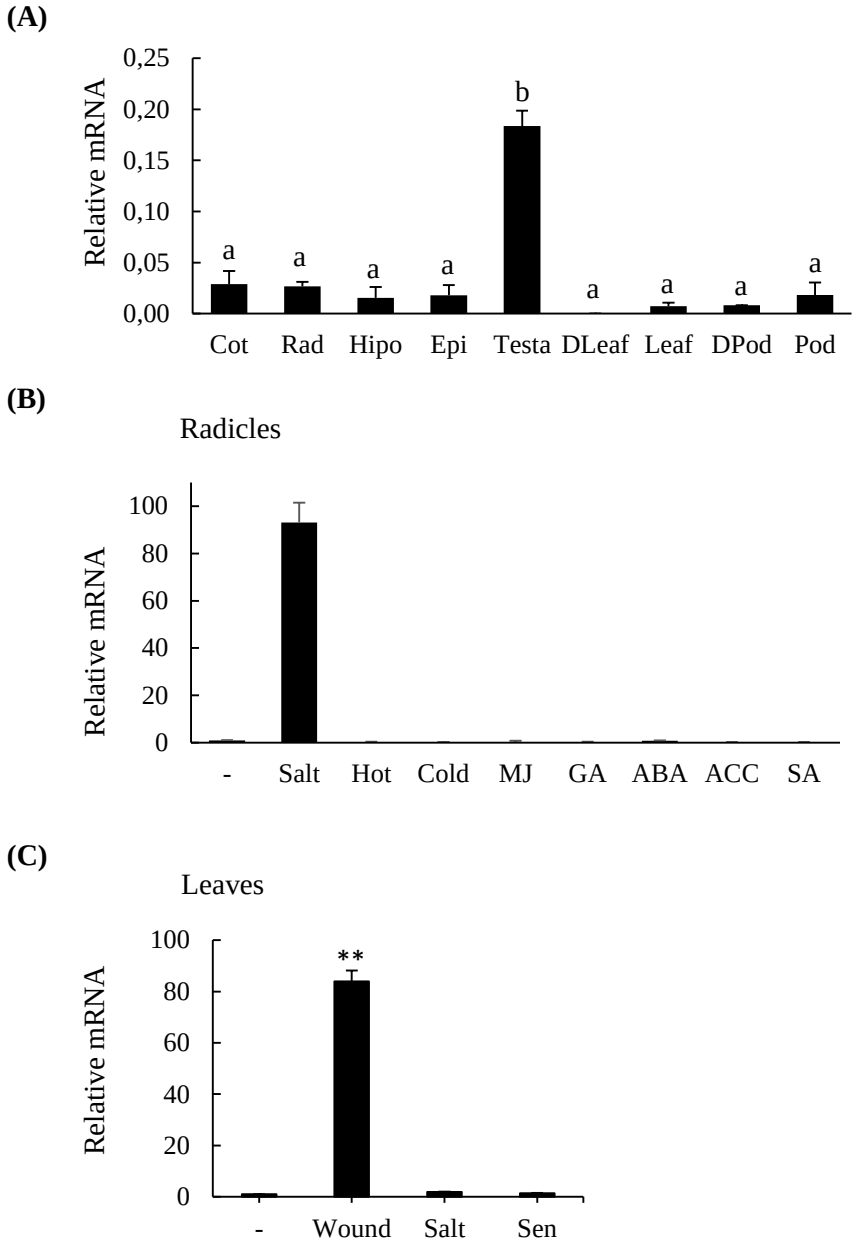

Figure 8. *Expression pattern of PvRNS3 in different tissues and physiological situations. (A) The relative expression level was determined in cotyledons (Cot), radicles (Rad), hypocotyls (Hypo), epitcotyls (Epi), the testa (Testa), developing leaves (DLeaf), mature leaves (Leaf), developing pods (DPod), and mature pods (Pod) and normalized using the geometric mean of the two reference genes (actin-2 and ubiquitin), and relative expression was calculated using the* $2^{-\Delta\Delta CT}$ *method (Livak and Schmittgen, 2001). (B) PvRNS3 expression was analysed in*

radicles from 6 DAI seedlings subjected to different stress situations (salt, cold, and heat) and treatments with different hormones (methyl jasmonate 250 µM, gibberellic acid 50 µM, abscisic acid 50 µM, salicylic acid 200 µM, 1-aminocyclopropane-1-carboxylic acid 10 µM) for the last 24 h. PvRNS3 was expression relativized as in (A) but the values were expressed relative to the expression of PvRNS3 in radicles from non-treated seedlings, which was considered the value 1. (C) PvRNS3 expression was analysed in wounded leaves after 24 h, in dark-induced senescing leaves after 6 days in continuous dark, and in plants irrigated with growth media supplemented with 50 mM NaCl. In all cases, the treatments were performed in first trifoliate leaves from plants at 21 DAI, as indicated in the Materials and Methods. PvRNS3 expression was relativized as in (A), but the values were expressed relative to the expression of PvRNS3 in the first trifoliate leaves obtained from non-treated plants, which was considered the value 1. Values are the mean ± SE of three biological replicates with three technical replicates. In (A), significant differences, according to Tukey's test, are indicated with different letters ($p \leq 0.05$). In (B, C), a t-test was carried out, and a significant value at $p \leq 0.01$ is indicated by two asterisks.

4. DISCUSSION

The T2 S-like ribonucleases in the common bean are composed of four members, for which expression has been analysed in several physiological processes. *PvRNS1*, *PvRNS2*, and *PvRNS4* increase their expression in cotyledons during the nutrient-mobilization phase (Diaz-Baena et al., 2021), whereas *PvRNS3* is induced in radicles after salt stress (Diaz-Baena et al., 2020). Among these genes, we have focused on the study of *PvRNS3*. The predicted protein encoded by *PvRNS3* maintains the eight conserved cysteines (Diaz-Baena et al., 2020), which are postulated to be involved in the formation of disulfide bridges (Thorn et al., 2012). The loss of activity obtained after DTT treatment suggests that the recombinant PvRNS3 purified and characterized in this study establishes these bridges. The analysis of the PvRNS3 sequence and its comparison with predicted Class I ribonucleases, both in the common bean and Arabidopsis, reveals that all genes have the same distribution of introns and exons, suggesting the same origin for all the members of this class of ribonucleases, and that the specialization in the function of each gene must have occurred after their divergence.

We have used the approach of heterologous expression of proteins in bacteria as a tool to purify PvRNS3 and characterize its enzymatic activity. Purified PvRNS3 is a functional ribonuclease that can use either rRNA or tRNA as a substrate and has negligible activity with DNA, either single or double-stranded. PvRNS3 shows higher activity at an acidic pH, which could be consistent with apoplastic or vacuolar localization, since these compartments maintain acidic pH levels at 5.0-5.5 (Feng et al., 2020). The extracellular localization of PvRNS3 can also be deduced from its sequence (Diaz-Baena et al., 2020). The high stability to heat denaturation shown by the purified is also remarkable. The inhibition observed with Cu and Zn is common to other T2 ribonucleases, such as the small ribonucleases from potato (Kumar et al., 2022) and pear (Hayashida et al., 2013), as well as the human RNase T2 (Thorn et al., 2012). This human RNase T2 has been crystallized, and a zinc-binding site has been predicted in its structure, although the enzyme does not require divalent ions as a cofactor (Thorn et al., 2012). It remains unclear whether zinc binding is of physiological relevance, but it seems to be a common characteristic among T2 ribonucleases. The expression of *PvRNS3* was higher in the testa than in any of the other tissues analysed. In addition, its expression was only induced after some stress situations, such as salt stress in radicles and wounding in leaves. This specific induction in some stress situations could indicate a specific role for PvRNS3 in these situations and not because of a general response to unfavorable situations in the common bean. The lack of induction under nutrient-mobilization situations, such as leaf or cotyledon senescence, also rules out a non-specific role of PvRNS3 in nucleic acid recycling under high mobilization situations. In plants, the ribonuclease T2 protein repertoire has expanded, and individual proteins have been adapted for a variety of functions. At least four genes are found in each plant genome that has been sequenced, and there is evidence of frequent duplications that have resulted in different numbers of ribonuclease T2 genes in different species (MacIntosh et al., 2020). This divergence of ribonuclease T2 families in plants implies the development of specific physiological roles for each member in the family. *AtRNS1*, a Class I gene, and *AtRNS2*, the only Class II gene, are the most studied ribonucleases in

Arabidopsis. AtRNS2 is thought to be involved in the degradation of rRNA in the vacuole (Floyd et al., 2017; Floyd et al., 2015). AtRNS1, like PvRNS3, has been predicted to be targeted to the secretory pathway and has been suggested to function in the processing of extracellular RNA in the apoplast (Borriego and Innes, 2023). In mammals, there is increasing evidence of the extracellular processing of RNA by extracellular ribonucleases (Tosar and Cayota, 2020). This may also be the case in plants, where the extracellular localization of multiple ribonucleases is hypothesized. The RNA used by these enzymes could be the result of release from damaged cells, although the existence of mechanisms involved in RNA secretion should be taken into account. In this way, it is also interesting to address the new potential functions of different classes of extracellular RNA during plant development or biotic interactions (Borriego and Innes, 2023). Regarding this extracellular action, it has recently been proposed that AtRNS1 is essential for the cell death in response to mycotoxin stress (Goodman et al., 2022). Furthermore, it has been demonstrated that extracellular ATP targets AtRNS1 to suppress this cell death process (Goodman et al., 2022). This can be triggered by total cellular degradation or by alternative mechanisms involving specific downstream gene regulation, for instance by acting on tRNA to produce a tRNA-derived fragment (Megel et al., 2019). Regarding this, it has been shown that T2 RNases from Arabidopsis (AtRNS1, AtRNS2, and AtRNS3) are essential ribonucleases for the production of tRNA-derived fragments (Megel et al., 2019). Although, as mentioned above, RNA degradation is assumed to take place mainly in the vacuole (Floyd et al., 2015; Floyd et al., 2017), further processing in the apoplast could be considered, as the accumulation of many rRNA fragments in the apoplast of Arabidopsis leaves has been described (Baldrich et al., 2019; Karim et al., 2022). The diversity of extracellular RNAs in plants has begun to be investigated, and the complexity that is beginning to be elucidated has led to the speculation that extracellular RNAs are involved in many functions in plant physiology, such as cell-to-cell communication or the stress response (Borriego and Innes, 2023).

The high level of expression of *PvRNS3* in the testa from the common bean is remarkable, although the significance of this ribonuclease during seed

germination and seedling development is still unknown. The seed coat has been usually considered a passive barrier protecting the embryo from harmful environmental conditions. However, in recent years, it has been proposed that it may play a more active role in germination, based on its inherent ability to store proteins that can remain active for many years (Raviv et al., 2017; Raviv et al., 2018). A proteome analysis of dead pericarps of *Anastatica hierochuntica* revealed the presence of hundreds of proteins released after hydration, including nucleases, chitinases, and proteins involved in the removal of reactive oxygen species (Khadka et al., 2020). We have shown the presence of several ribonuclease activities in the testa as early as 24 h after the start of imbibition, and that the enzymes remain active after radicle protrusion. More interestingly, RNA encoding *PvRNS3* was increased in the testa at 3 DAI compared to at 1 DAI suggesting a functional role for this ribonuclease in seedling establishment.

We propose that the testa surrounding the embryos not only provides a physical barrier for embryo protection, but also stores and synthesizes active enzymes and, probably, metabolites. The release of these components into the surrounding environment after seed hydration could help germination and seedling establishment, creating a favorable environment for seedlings. This fact is important considering the sessile nature of plants, and the release of substances or enzymes from the seeds upon hydration could provide advantageous situations for developing seedlings. The secreted enzymes may be intended to degrade external molecules to provide nutrients to developing seedlings and to protect germinating seeds from soil pathogens. Considering that the testa is a maternally derived tissue, it will be interesting to address if the exposure of the mother plants to stresses during the seed filling phase affects the testa constituents and if these changes affect the viability of seeds during germination and seedling development. It will also be interesting to find out if the storage of seeds and its conditions affect the composition of the testa and the viability of seeds. It will be very interesting to determine how the enzymatic and nucleic acid composition in the testa is affected by these conditions and if these changes affect the establishment capacity of the new seedling. These would contribute to improvements in agricultural practices, with a better knowledge of nucleic acid

metabolism in the near future being a central role in achieving this important task.

5. SUPPLEMENTARY MATERIAL

A

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  1-  MESKNSILVKLLLLLHYLSLFCASQDFDFFYFVQQWPGSFCDTEKSCCYP
 51-  TSGKPNADFGIHGLWPNSNDGSFPSNCDPNNPFNPSQISDLTSSLESNWP
101-  TLACPSGDGMTFWSHEWTKHGTCSESVLKQHDYFEAALSLRQKANLLQAL
151-  TTAGIQPNGGSYSLSSIKGAIKDAIGFTPFIECNVDSSGNSQLYQVYLCV
201-  NTSGSDFIDCSVFPRGKCGSNIEFPSF
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B

RNS3	
Length	227 aa
Molecular Weight (with/without signal peptide)	24,9 kDa / 22,1 kDa
Extinction coefficient (280 nm)	37.930 M^{-1} cm^{-1}
Isoelectric Point (pI)	4,47
Charge at pH 7	-14,48
Negatively charged residues (Asp/Glu)	21 (9,25 %)
Positively charged residues (Arg/ Lys)	12 (5,29 %)

Figure S1. *Characterization of PvRNS3 protein sequence. A. Amino acid sequence deduced for PvRNS3. The predicted signal peptide is underlined. B. Main properties of the amino acid sequence.*

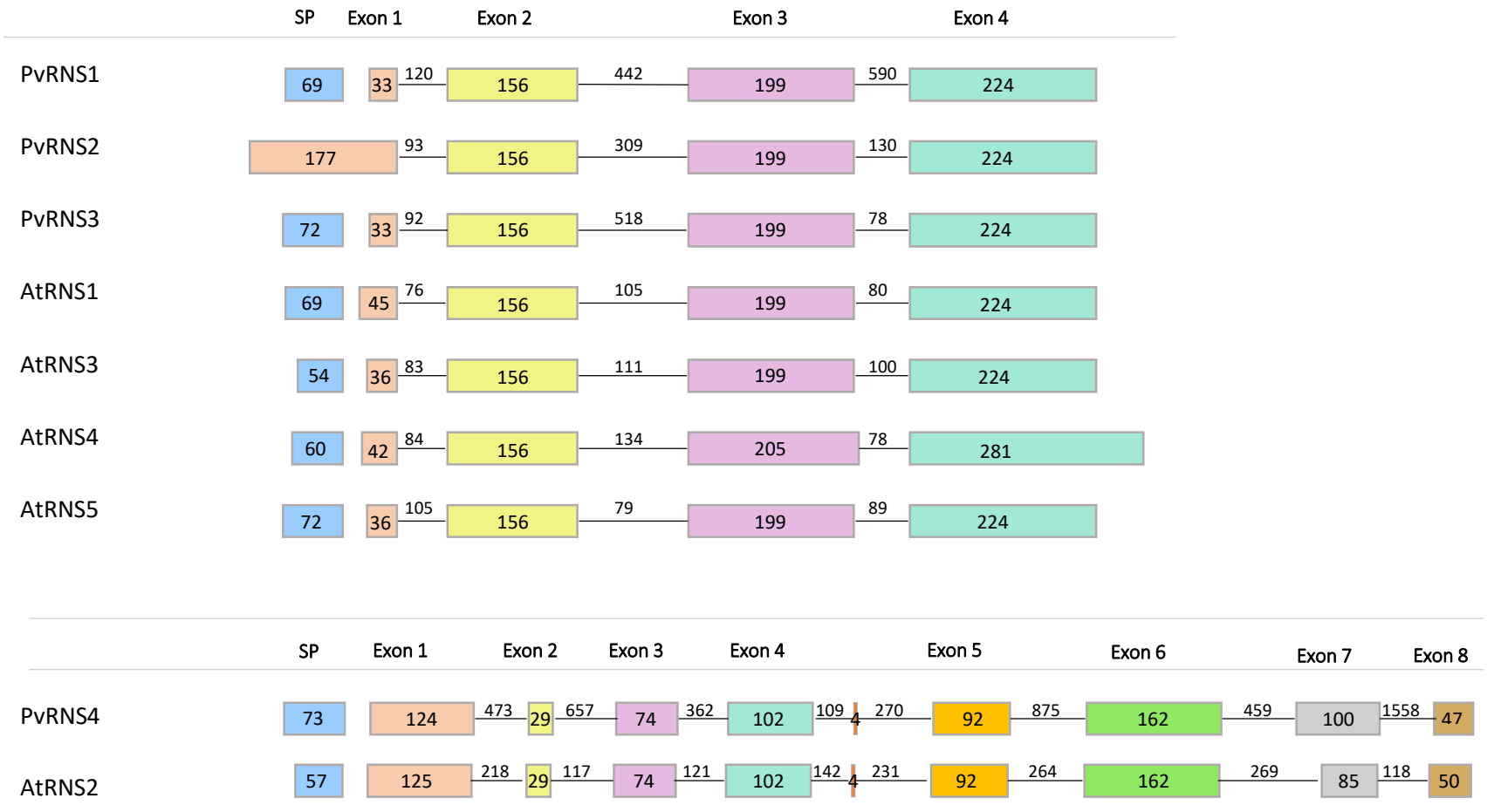

Figure S2. *Comparison of exon/intron gene structure in Phaseolus vulgaris and Arabidopsis thaliana. SP represents signal peptide structure. The numbers indicate the length of either exons or introns. The accession number and chromosome are indicated.*

Table S1. Primers used in this study.

Primers used for qRT-PCR:

Gene	Accession number	Sequences 5'>3'
PvRNS1	Phvul.002G084500	F: CTGAGCTGCCCAAGTAGCAA R: CTTTGATCAAGCTCAGATTGTGC
PvRNS2	Phvul.002G170800	F: CCAAGTGGTAACGGGGTCCA R: GGTTGGCTCTCTGCTTCAGATC
PvRNS3	Phvul.002G084600	F: GGACTCATCTGGGAACAGCC R: GGGGAACACAGAGCAGTCAA
PvRNS4	Phvul.010G110200	F: ATCCCCTTGGAGGCATTATC R: AGCATAGGCGAAGTTCCTCA
Actin	Phvul.006G209800.1	F: GCAATTCAGGCTGTCTTGTCTTTGT R: TAAATCACGGCCAGCAAGATCC
Ubiquitin	Phvul.007G270100.1	F: TACATGCGATCTTGGACTGGC R: GGGGCTTTTCTGGGTAGTCT

Primers used for qRT-PCR:

Gene	Sequence 5'>3'
PvRNS3	F: GTCGACCAGGATTTTGATTTCTTCTA R: CTCGAGAAAAGATGGGAACTCAATGT

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4. GENERAL DISCUSSION.

Nucleic acids are polymers of nucleotides, and therefore, they contain nitrogen and phosphorus in their composition. In situations of high energy demand, such as tissue growth and development or response to various stresses, nucleic acids could play a crucial role as a reservoir of nutrients, supplying the essential elements necessary for the proper function of the plant (Luhtala et al., 2010). RNA, being the most abundant nucleic acid in most tissues and conditions (Veneklaas et al., 2014), would have a relevant role in this supply of nutrients. In fact, it has been shown an increase in RNAse activity in nitrogen and phosphate starvation (Luhtala et al., 2010).

Climate change is expected to increase the impact of abiotic stresses, which is highly dependent on environmental factors (AbdElgaward et al., 2016). Additionally, nutrient availability plays a crucial role in ensuring plant viability. The work developed in this thesis contributes to the knowledge of the diverse mechanisms involved in plants subjected to conditions that require a significant demand for nutrients. The use of genetic modifications to enhance these mechanisms can lead to plants more tolerant to such conditions and with enhanced efficiency in nutrient utilization. The enhancement of genes encoding extracellular RNases can serve as a valuable tool in the genetic improvement, making the plant more efficient at nutrient recycling (Sugawara et al., 2016).

At the molecular level, the response to high-nutrient-demand situations requires the collective action of several gene groups (Kumar et al., 2020). There are relatively few plants resistant to abiotic stress available for commercialization in comparison to those resistant to biotic stresses given the complexity of genetic modification in abiotic response pathways. However, despite these difficulties, genetic engineering will overcome the limitations of traditional field methods (Hussain et al., 2021).

This doctoral thesis has been focussed on the involvement of nucleic acids as nutrient reservoirs in plants during high nutrient demand situations. T2 ribonucleases are the most abundant ribonucleases in plants (MacIntosh et al., 2020). This fact, along with previous work that identified the T2 ribonuclease family in common beans (Diaz-Baena et al., 2020) prompted the choice to this

family for the current study. Plant T2 ribonucleases are classified into three classes (Igic & Kohn, 2001). It is postulated that Class I is related to stress response, Class II to cellular homeostasis maintenance, and Class III to gametophytic incompatibility. This thesis has focused on the study of Class I ribonucleases (comprised of the genes *PvRNS1*, *PvRNS2*, and *PvRNS3*) and Class II (formed by the gene *PvRNS4*), collectively known as T2-S like ribonucleases (Gho et al, 2020; Díaz-Baena et al., 2020). It is postulated that the four genes encode functional ribonucleases as they contain the two conserved histidines in the essential CAS I and CAS II motifs required for catalytic activity (Irie et al., 1999).

The role of common bean S-Like T2 RNases was analysed during cotyledon ontogeny (Chapter 1), and it has been demonstrated that different ribonucleases are induced in cotyledons during the nutrient mobilization phase in seedling development. This evidence that S-like T2 RNases are involved in the turnover of RNA in the cotyledons during this phase. The study of nucleic acid degradation during the seed filling phase in developing fruits (Chapter 2), revealed a significant role of seed coat in nucleotide metabolism during this phase. This can be deduced from the high enzymatic activity and expression of some genes involved in nucleotide metabolism during the seed filling stage. The high level of ribonuclease activity in seed coats observed during the seed filling phase (Chapter 2) prompted us to analyse this activity in the testa from germinating seedlings. We found high ribonuclease activity in this tissue and high expression of *PvRNS3*, suggesting an additional role for the testa apart from forming a protective barrier for the embryo (Chapter 3). The high expression of *PvRNS3* in this tissue as well as in seed coats from developing seeds (Chapter 2) and in salt-treated radicles (Diaz-Baena et al., 2020) prompted us to focus on the characterization of this enzyme. Thus, PvRNS3 was overexpressed and purified as recombinant protein in *Escherichia coli* and the purified protein was further characterised (Chapter 3). The enzyme showed specific activity for RNA, confirming that *PvRNS3* codes for a functional ribonuclease.

Nucleic acid degrading activities were determined in the physiological processes described above and, in all these conditions the RNA degrading activity was always higher than the DNA degrading activity. In the conditions studied, three ribonucleases with relative mobilities of 19 KDa, 17 KDa, and 16 KDa were identified, which coincide with those previously identified in common bean radicles (Diaz-Baena et al., 2020). However, it is important to realise that these values may not correspond with the real molecular weights of the enzymes, as the electrophoresis has been carried out under non-denaturing conditions to allow to determine their enzymatic activity in the in-gel assay. In the cotyledons, the 19 KDa and 17 KDa ribonucleases increased their activity as the cotyledons mature, while the 16 KDa decreased its activity with cotyledon aging. Induction of nuclease activities have been described as well during cotyledon senescence (Lambert et al., 2016). The induction of both activities, nuclease and ribonuclease, coincides with the phase of high reserve protein mobilisation in cotyledons (Quiles et al., 2009), suggesting its involvement in nutrient mobilization. However, the optimal pH determined for nucleases and ribonucleases was different; nucleases showed higher activity at neutral pH (Lambert et al., 2016), whereas the ribonuclease showed higher activity at acidic pH (Chapter 1). This suggests that nucleases and ribonucleases have specific functions or contribute differently to certain aspects of this process. During fruit development, the total ribonuclease activity was high in pod valves and seed coat but barely perceptible in the embryo. In the pod valves, the 19 KDa protein was detected, while in the seed coat, the 17 KDa and 16 KDa proteins were found (Chapter 2). The three ribonucleases were present as well in the testa from germinating seedlings, with the 16 KDa one being the most abundant ribonuclease (Chapter 3). This ribonuclease is the same induced in radicles of common bean plants exposed to salt stress (Diaz-Baena et al., 2020).

In this study, we showed correlation between some ribonuclease activities and the expression of some ribonuclease genes. However, the unequivocal association of each ribonuclease to each activity band requires further identification of the enzyme responsible for each of the activities detected in the gel enzyme assays. Nevertheless, comprehensive analysis of the expression

patterns of the four genes belonging to the S-like T2 RNases has been conducted under the studied physiological conditions. The expression of the genes encoding S-like T2 ribonucleases during cotyledon ontogeny showed a similar trend than the activities determined in these conditions, with all the genes, except for *PvRNS3*, increasing its expression (Chapter 1). These data contribute to highlight the importance of nucleotide metabolism during germination and seedling development in common bean. In these processes, it has been reported high enzymatic activity and gene expression for nucleases (Lambert et al., 2014; 2016), nucleotidases (Cabello-Diaz et al., 2012; 2015) and nucleosidases (Delgado-García et al., 2021), all enzymes related with nucleotide metabolism.

The four genes (*PvRNS1 to PvRNS4)* belonging to S-like RNases, were expressed in valves and seed coats during common bean seed filling process. *PvRNS1* was expressed mainly in pod valves, *PvRNS3* in the seed coat, and *PvRNS4* showed constitutive expression in both tissue (Chapter 2). *PvRNS4* is the only gene belonging to Class II in common bean, what seems to be common in plants (Igic and Kohn, 2001; Köthte and Kohn, 2011; Zhu et al., 2020). It is postulated that PvRNS4 is a vacuolar enzyme and performs the same homeostasis maintenance function assigned to AtRNS2 (Diaz-Baena et al., 2020), which needs vacuolar/lysosomal localization to carry out its function (Hillwig et al., 2011).

In testa from germinating seedlings, only *PvRNS3* and *PvRNS4* were detected. The seed coat constitutes the interface between the internal structures of the seed and the external environment. Its composition plays a pivotal role in determining seed germination, vitality, and potential longevity (Souza et al., 2001). Nucleic acid degradation activity was observed in the seed coat after seed hydration and this activity persisted during root growth. This period of activity coincides with a phase in which the cotyledons experience significant nutrient mobilization (Quiles et al., 2009). This leads us to reconsider the conventional idea that the seed coat has mainly a protective function, but that, in addition, the release of proteins stored in this tissue could be a fundamental factor for the correct and successful germination of the seed. In recent years, it has been suggested that this

tissue stores proteins that remain inactive for extended periods (Godwin et al., 2017). Latter, when the seed is imbibed, these stored proteins allow the plant to initiate its development very rapidly without the need for immediate protein synthesis. In this regard, it has already been shown that, after imbibition, the plant requires a rapid activation that implies the use of various stored compounds such as mRNAs (Zhao et al., 2020).

Among the genes analysed, *PvRNS3* is highly expressed in the developing seed coat as well as in the testa of seedlings (Chapter 2 and 3). Moreover, it is the only ribonuclease gene induced in radicles exposed to saline stress (Diaz-Baena et al., 2020), and its expression is also induced in other abiotic stress situations, such as in wounded leaves (Chapter 3). The overexpression of *PvRNS3* and the characterization of the recombinant protein showed that it encodes a functional ribonuclease with an acidic pH optimum, which is consistent with its predicted extracellular location. The activity showed high thermostability and inhibition by Zn and Cu which is common among ribonucleases (Kumar et al., 2022). The corresponding activity of the 16 KDa ribonuclease protein and the expression of *PvRNS3* in all the situations analysed in this study would support that *PvRNS3* could encode the 16 KDa ribonuclease. However, a more comprehensive study is needed to correlate activity and gene.

Besides their significance for plant biology, ribonucleases have very important medical applications. Human RNase T2 plays a role in regulating the innate immune response against bacteria, non-phagocytic helminth parasites, and viruses, and has also been shown to have antitumour actions (Bruno et al., 2022). The antiproliferative and antitumour effects have been demonstrated in vivo in athymic mice bearing human melanoma tumours, in which administration of a plant ribonuclease caused a significant reduction in tumour size (Matuosek et al., 2010). Nevertheless, while these findings are promising, further studies and trials are required to determine their efficacy and safety.

In general terms, the research presented in this doctoral thesis has showed the relevance of nucleic acids as a crucial source of nutrients in high-demand situations. Particular emphasis has been placed on the role of common bean

ribonucleases T2 and their contribution to various plant development processes and stress responses. The knowledge of an efficient mobilization of nucleic acids can provide the necessary insights to improve the quality of seeds and other agronomic aspects, often with their role as a nutrient reservoir being underestimated.

These findings open exciting future investigations, it would be necessary to develop techniques that allow establishing a correlation between the enzymatic activity of ribonucleases and their gene expression. This approach would allow a deeper exploration of the functionality of each ribonuclease in different contexts and conditions. These advancements are essential to better understand how plants manage their resources and adapt to constantly changing environments.

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6. CONCLUSIONS.

1. The increased expression of genes encoding T2 S-like ribonucleases in senescent cotyledons suggests that these enzymes are involved in the mobilization of nucleic acids during the post-germinative development of common beans.

2. The enzymatic activities and the gene expression pattern of T2 S-like ribonucleases suggest high nucleotide metabolism in fruits during seed filling stage with an especial involvement of seed coat in the process.

3. The presence of nucleotidase, nucleosidase and allantoinase activities in both maternal tissues (valves and seed coats) indicate that some of the nucleotides are fully degraded before transferring to the embryos. In addition, nucleic acid degrading activities in embryos were very little supporting the fact that this part of the seed is involved in the accumulation of storage reserves.

4. The presence of all essential residues in the sequence of PvRNS3 and the detection of ribonuclease activity with the purified protein lead to the conclusion that *PvRNS3* encodes a functional acidic ribonuclease.

5. The coincidence of *PvRNS3* expression and the16 kDa protein activity patterns suggests that this is the enzyme encoded by *PvRNS3*.

6. The fact that *PvRNS3* is induced under some stress situations but not under nutrient mobilization situations suggests a role for this protein different to nucleic acid recycling in mobilization processes.

7. *PvRNS4* was detected in all tissues analysed supporting the idea that its expression is constitutive.