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High Ribonuclease Activity in the 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 suggest 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

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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 hydrolyze single-stranded RNA, forming oligo- or mononucleotides with a terminal 3′-phosphate [1], and they have been classified as RNase A, Rnase T1, and RNase T2 [2].

Ribonucleases belonging to the T2 family are the most widely distributed and have been described in bacteria, fungi, and eukaryotes [2]. 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 [2]. This family has two conserved active sites, CAS I and CAS II. Each domain contains a highly conserved His residue that is catalytically important [3]. Mutations of these histidine residues lead to inactivation of the enzyme [1]. The structure of human RNase T2 has four disulphide bridges [4], which are conserved in most of the plant RNases [5]. The enzymatic reaction occurs in two steps, transphosphorylation and hydrolysis, with the conserved histidine being required in both steps [2].

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 [4],

and the same happened in *Drosophila melanogaster* [6]. 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 [7]. For instance, the RNase T2 family is composed of five members in Arabidopsis [8], eight in rice [9], thirteen in common bean [5] and soybean [10], and twenty-one in *Eucommia ulmoides* [11]. 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 [12]. 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 [7]. 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 [5,11,13,14].

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 [7]. Induction of the expression of some Class I genes has been described in plants subjected to stress or Pi starvation [7]. Some of the members of this class have been hypothesized to be secreted from cells [7], suggesting that these ribonucleases could be involved in the degradation of apoplastic RNA [15]. 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 [16]. The diversity of extracellular RNAs found in the plant apoplast and their possible secretion mechanism have been recently reviewed [16]. In addition, recent studies also reveal that some members of the Class I T2 ribonucleases can be involved in the salvage of intracellular RNA [15].

Class II is proposed to function in maintaining normal cellular homeostasis by recycling rRNA and typically contains a single gene per genome [7]. RNS2 is the Class II enzyme from Arabidopsis, which is mainly located in the vacuole [17]. RNS2 has been shown to be required for normal rRNA turnover [17,18]. The enzymatic reaction carried out by this enzyme is essential for maintaining normal cellular levels of nucleotides/nucleosides via RNA turnover [7], and rns2 mutants have a deficiency in rRNA degradation leading to a decrease in cytoplasmic nucleoside and nucleotide concentrations [19]. It has been hypothesized that this role in RNA salvage is the ancestral function of ribonuclease T2 enzymes [7].

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 [7].

In common bean, the S-like ribonuclease T2 subfamily is composed of four genes named *PvRNS1* to *PvRNS4* [5]. PvRNS4 is the only T2 ribonuclease in common bean belonging to Class II, whereas the others three belong to Class I [5], and therefore, they are postulated to be involved in a variety of stress responses [7]. 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 [5]. 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 [5].

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 [20]. 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 analyzed 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 [5]. 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 [21].

The plant materials to analyze 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 [21].

All plant material was wrapped in aluminum 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 Extract 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 15,000× *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 [22], with bovine serum albumin as the 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. [5].

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-Na_2) 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 catalyzed 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 [20]. The cDNA was prepared from total RNA as previously described [20].

2.6. Quantitative Real-Time PCR

Relative gene expression was determined via quantitative real-time PCR, as indicated previously [5], 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 [23]. 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 *Sal*I and *Xho*I (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 *Sal*I and *Xho*I, 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 the means of at least three biological and two technical replicates. Values are the mean ± SE. The analyses performed are indicated in the legends 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 analyzed 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 analyzed. 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).

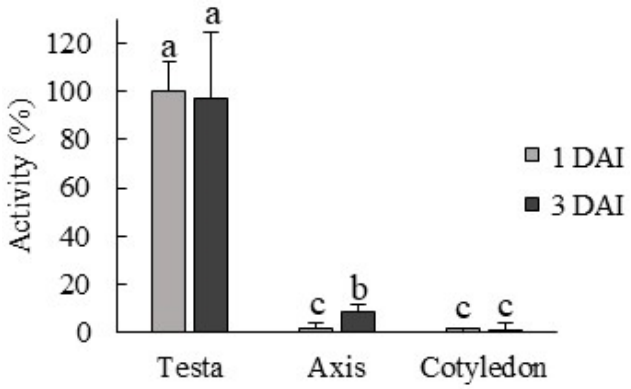

Figure 1. In vitro ribonuclease activity in the testa, embryonic axis, and cotyledons from seedlings at 1 and 3 days after start of imbibition. The specific ribonuclease activity was normalized to 100% according to the specific activity obtained for the testa at 1 DAI. The activities are the mean ± SE 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.

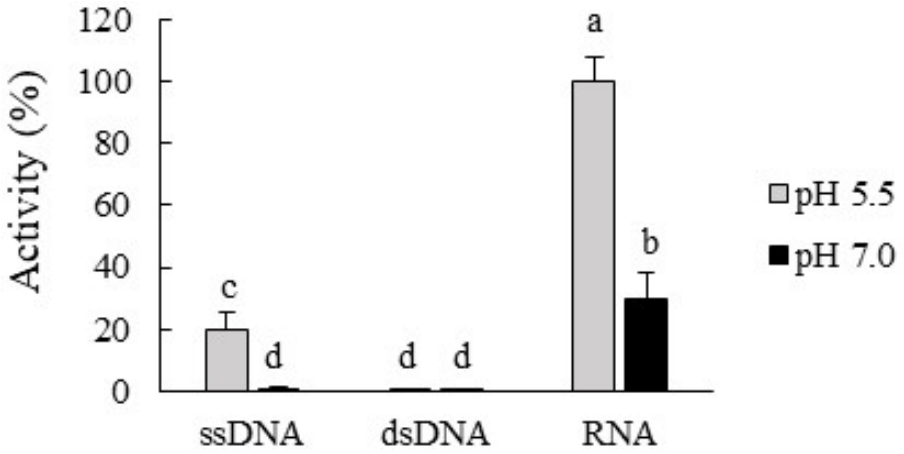

Figure 2. In vitro nucleic-acid-degrading activity with ssDNA, dsDNA, or RNA as substrates in crude extracts obtained from the testa at 3 DAI. The activity was determined both at pH 5.5 and 7.0. The specific activity was normalized to 100% according to the specific activity obtained for RNA and pH 5.5. 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).

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).

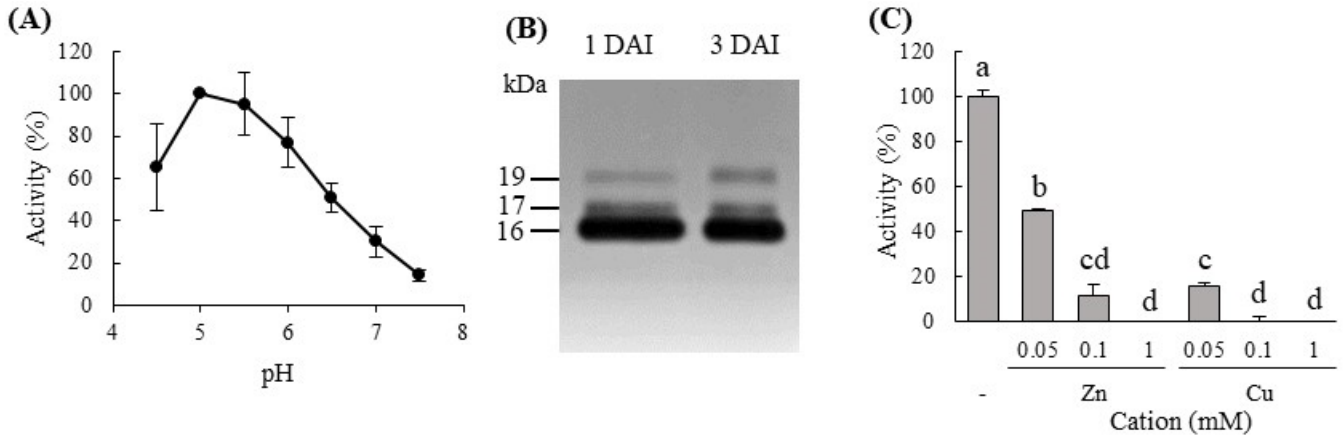

Figure 3. (**A**) The activity in crude extracts from the testa obtained from seedlings at 3 days after imbibition was determined at the pH indicated. The activity was normalized to 100% according to the highest value. The activities are the mean ± SE of three independent determinations. (**B**) In-gel 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 ($p \leq 0.05$).

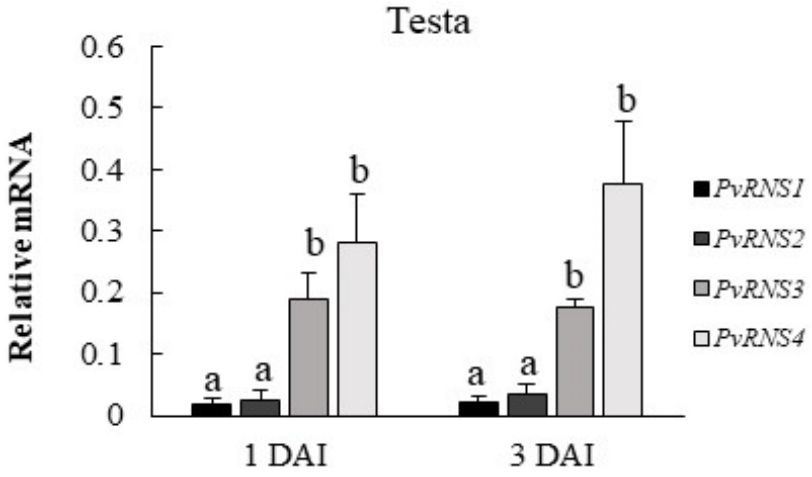

Figure 4. Expression pattern of S-like T2 ribonucleases in the testa from common bean seedlings at 1 and 3 days after imbibition. The relative expression level was normalized using the geometric mean of the two reference genes (*actin-2* and *ubiquitin*), and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method [23]. 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 [5]. 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).

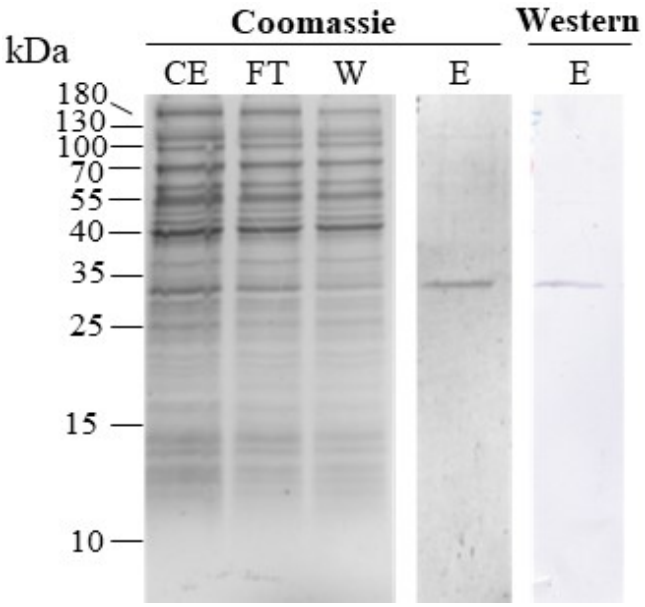

Figure 5. Purification of recombinant PvRNS3 overexpressed in *E. coli*. The purification procedure is described in the Materials and Methods section. The following samples were loaded in each lane of the polyacrylamide gel: crude extract (CE), proteins not bounded to the column and collected as the 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 analyzed 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.

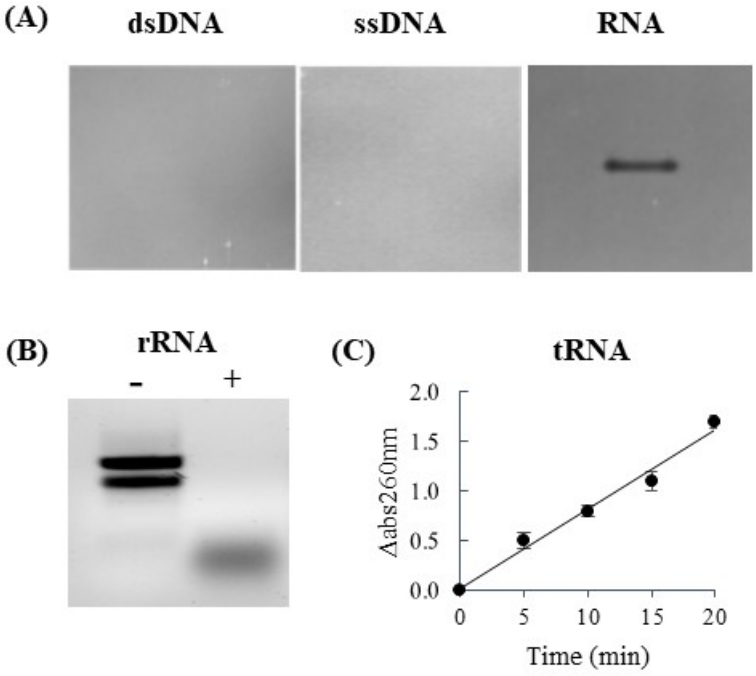

Figure 6. Substrate specificity of purified PvRNS3. (**A**) Nuclease and ribonuclease activities assayed with double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), or total RNA (RNA) using the in-gel assay. (**B**) Ribonuclease activity assayed with rRNA using the agarose gel assay. Lane −: reaction 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 Protein

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). 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).

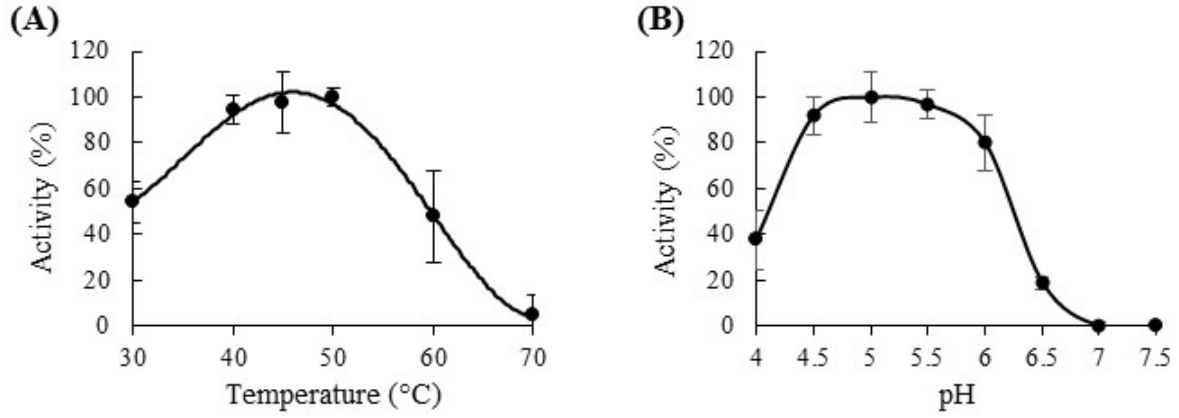

Figure 7. *Cont.*

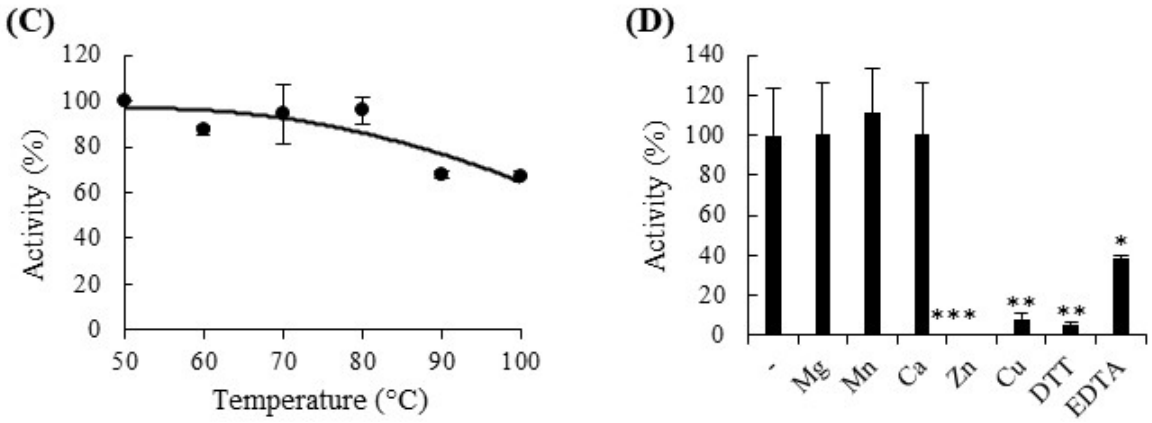

Figure 7. Characterization of ribonuclease activity from purified PvRNS3. In all the cases, the activity was assayed with RNA from the torula using the in vitro activity assay. (**A**) To establish the optimum temperature, the activity was assayed at the indicated temperature for 30 min. (**B**) To stablish 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.

3.5. Molecular Characterization of PvRNS3 Gene

The expression level of *PvRNS3* was analyzed 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).

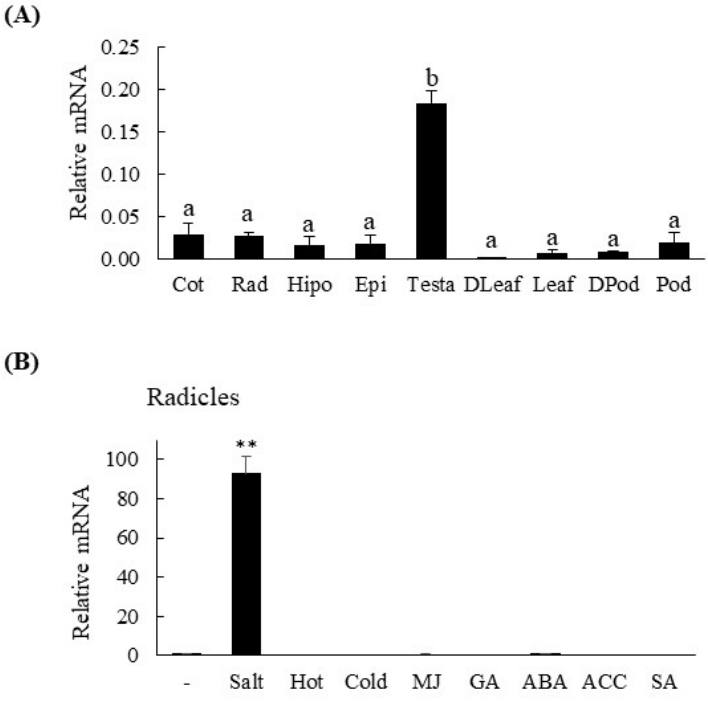

Figure 8. *Cont.*

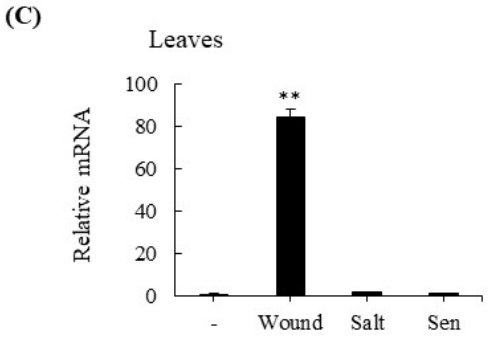

Figure 8. Expression pattern of *PvRNS3* in different tissues and physiological situations. (**A**) The relative expression level was determined in cotyledons (Cot), radicles (Rad), hypocotyls (Hypo), epitcotyls (Epi), the testa (Testa), developing leaves (DLeaf), mature leaves (Leaf), developing pods (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 [23]. (**B**) *PvRNS3* expression was analyzed 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 analyzed 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.

We analyzed 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 analyzed (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 [5], and that was included in the analysis as the positive control.

We also wanted to analyze 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.

4. Discussion

The T2 S-like ribonucleases in the common bean are composed of four members, for which expression has been analyzed in several physiological processes. *PvRNS1*, *PvRNS2*, and *PvRNS4* increase their expression in cotyledons during the nutrient-mobilization phase [20], whereas *PvRNS3* is induced in radicles after salt stress [5]. Among these genes, we have focused on the study of *PvRNS3*. The predicted protein encoded by *PvRNS3* maintains the eight conserved cysteines [5], which are postulated to be involved in the formation of disulfide bridges [4]. 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 [24]. The extracellular localization of PvRNS3 can also be deduced from its sequence [5]. 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 [25] and pear [26], as well as the human RNase T2 [4]. 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 [4]. 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 analyzed. 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 [7]. 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 [18,27]. 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 [16]. In mammals, there is increasing evidence of the extracellular processing of RNA by extracellular ribonucleases [28]. 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 [16]. Regarding this extracellular action, it has recently been proposed that AtRNS1 is essential for the cell death in response to mycotoxin stress [29]. Furthermore, it has been demonstrated that extracellular ATP targets AtRNS1 to suppress this cell death process [29]. 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 [30]. 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 [30]. Although, as mentioned above, RNA degradation is assumed to take place mainly in the vacuole [18,27], further processing in the apoplast could be considered, as the accumulation of many rRNA fragments in the apoplast of Arabidopsis leaves has been described [31,32]. 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 [16].

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 [33,34]. 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 [35]. 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.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13112750/s1, Figure S1: Characterization of PvRNS3 protein sequence; Figure S2: Comparison of exon/intron gene structure in *Phaseolus vulgaris* and *Arabidopsis thaliana*. Table S1: Primers used in this study.

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