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ORIGINAL ARTICLE

Nucleotide metabolism in common bean pods during seed filling phase reveals the essential role of seed coats

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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 for a correct communication between the different seed compartments. Nucleotides are essential components with nitrogen and phosphorous on its molecules, and its 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

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 colors and patterns.

The pods that surround the seeds have an important and dual role during seed formation such 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

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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 developed, 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 makes 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 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. 2012). 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. 2012). Thus, nucleic acids may play a crucial role in processes involving nutrient mobilization. In wheat plants subjected to N starvation, it 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 an essential role for seed formation; however, the research carried out so far on nutrient mobilization in pods is very scarce, specially 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 catalyzed by the enzymes studied in this work is shown in Fig. 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.

Materials and methods

Plant growth

Common bean (*Phaseolus vulgaris* L. Great Northern) seeds were sterilized 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 in medium supplemented with 10 mM nitrate (Gálvez-Valdivieso et al. 2013) and material was collected as indicated above.

Preparation of crude extracts

Frozen powder was homogenized 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 15,000 g for 10 min at 4 °C, the supernatants were considered as crude extracts.

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.

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 min. 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 increases 1 unit 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.

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 catalyzing the production of 1 µmol of phosphate per minute.

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 catalyzing the production of 1 µmol of xanthine per minute.

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 catalyzing the production of 1 µmol of allantoic acid per minute.

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).

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.

Statistical analyses

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

Results

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 (Fig. 1). 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 (Fig. 2). With DNA as substrate, the specific activity was mainly determined in seed coats both with dsDNA (Fig. 2a) and ssDNA (Fig. 2b), the activity being higher with ssDNA than with

Fig. 1 Photographs of French bean pods during seed filling stage. **a** Representative whole pod. **b** Pod opened longitudinally. **c** The three tissues analyzed 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

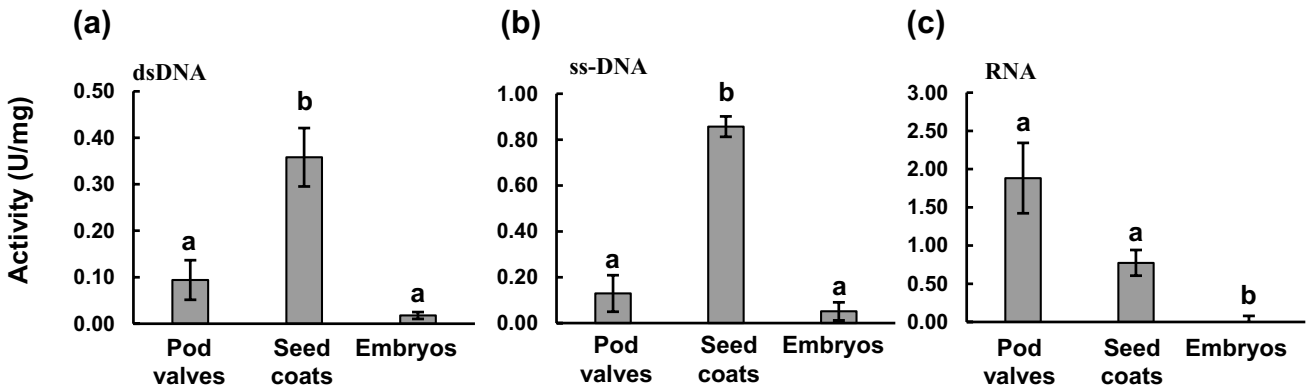

Fig. 2 In vitro nucleic-acids-degrading activities in common bean pods separated in valves, seed coats, and embryos during seed filling stage. Nucleic-acid-degrading activities with dsDNA (**a**), ssDNA (**b**) or RNA (**c**) were determined in vitro using crude extracts obtained from each tissue. Values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

dsDNA. With RNA as substrate, the specific activity was high in valves and seed coats and undetectable in embryos (Fig. 2c).

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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 3b).

The expression of the genes of the S1 nuclease and S-like T2 ribonuclease families were analyzed 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 (Fig. 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 (Fig. 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 (Fig. 4b). *PvRNS1* was more expressed in valves and *PvRNS3* in seed coats than in other tissues. *PvRNS4* was equally expressed in the three tissues (Fig. 4b).

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 (Fig. 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 (Fig. 5).

In addition, the expression of 11 genes belonging to the HAD family was analyzed (Fig. 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 the genes expressed in any part of the pods (Fig. 6). *PvNTD9* was expressed specifically in seeds, both in seed coats and embryos, being the gene with higher expression

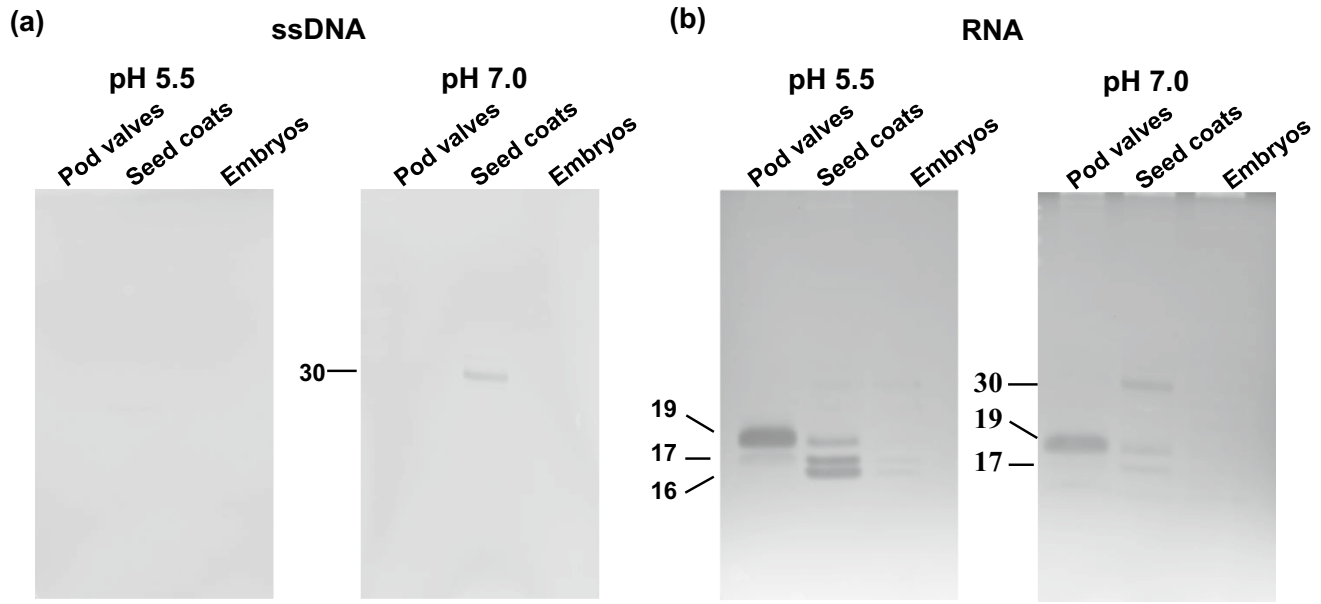

Fig. 3 In-gel assays of nuclease and ribonuclease activities. In-gel assays with ssDNA (**a**) or RNA (**b**) as substrates were performed at pH 5.5 and 7.0 using crude extracts obtained from valves, seed coats, and embryos from pods at the seed filling stage. The predicted molecular masses for the protein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

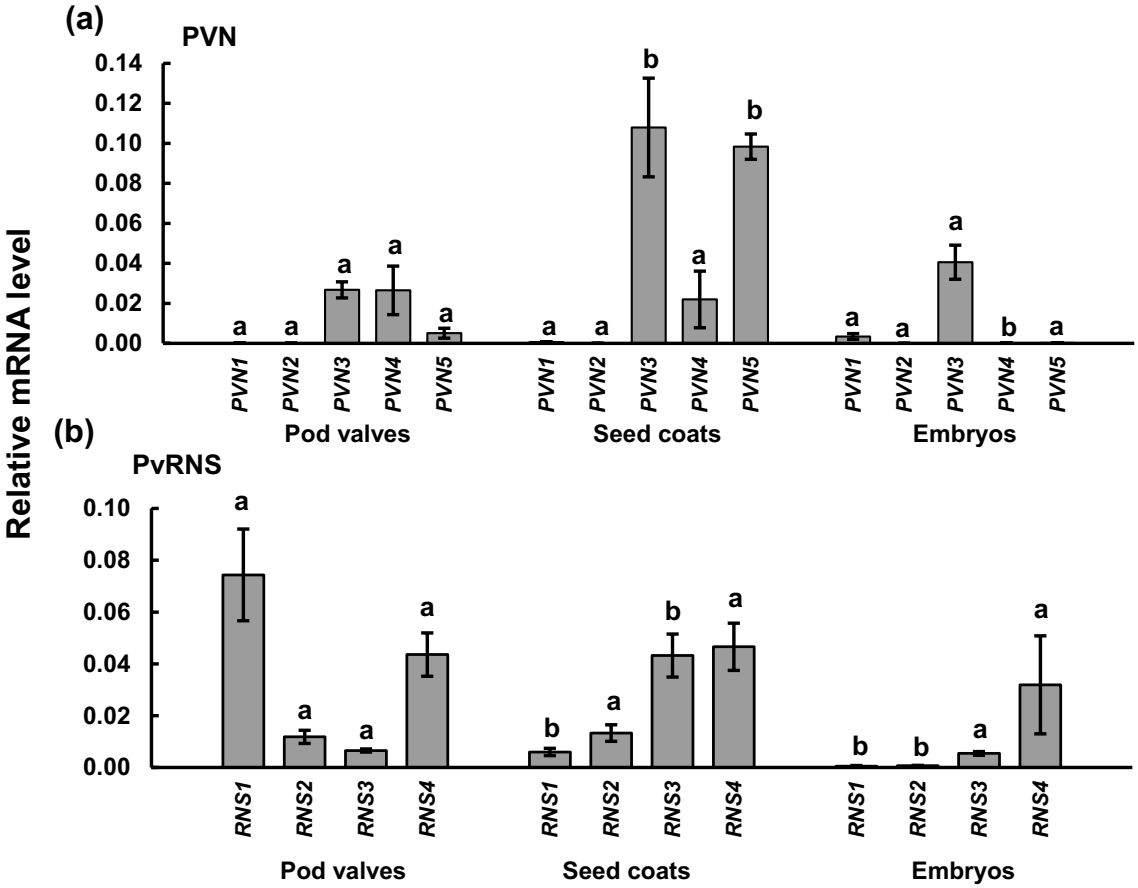

Fig. 4 Expression pattern of S1 nucleases and S-like T2 ribonucleases in pods of common bean. S1 nucleases (*PVN1* to *PVN5*) (**a**) and S-like T2 ribonucleases (*PvRNS1* to *PvRNS4*) (**b**) expression analysis was performed using qRT-PCR on total RNA samples extracted from valves, seed coats, and embryos of common bean pods at seed filling stage. Results were normalized with the geometric mean of actin-2 and ubiquitin genes and analyzed using the $2^{-\Delta CT}$ method. Values are expressed as mean ± SE of three independent biological samples with two technical replicates. Different letters indicate significant differences among tissues for the same gene as analyzed by ANOVA followed by Tukey's post hoc analysis ($p \leq 0.05$)

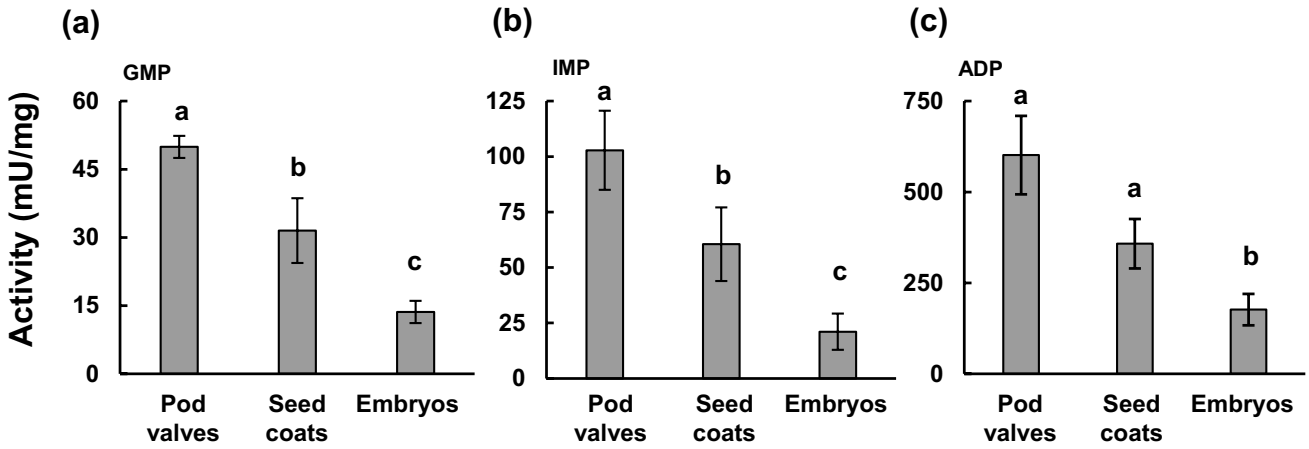

Fig. 5 Nucleotidase activity with different nucleotides. The activity was tested with GMP (**a**), IMP (**b**), and ADP (**c**) as substrates in crude extracts obtained from valves, seed coats, and embryos of common bean pods at seed filling stage. Means ± SEs of three independent biological samples with two 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 ($p \leq 0.05$)

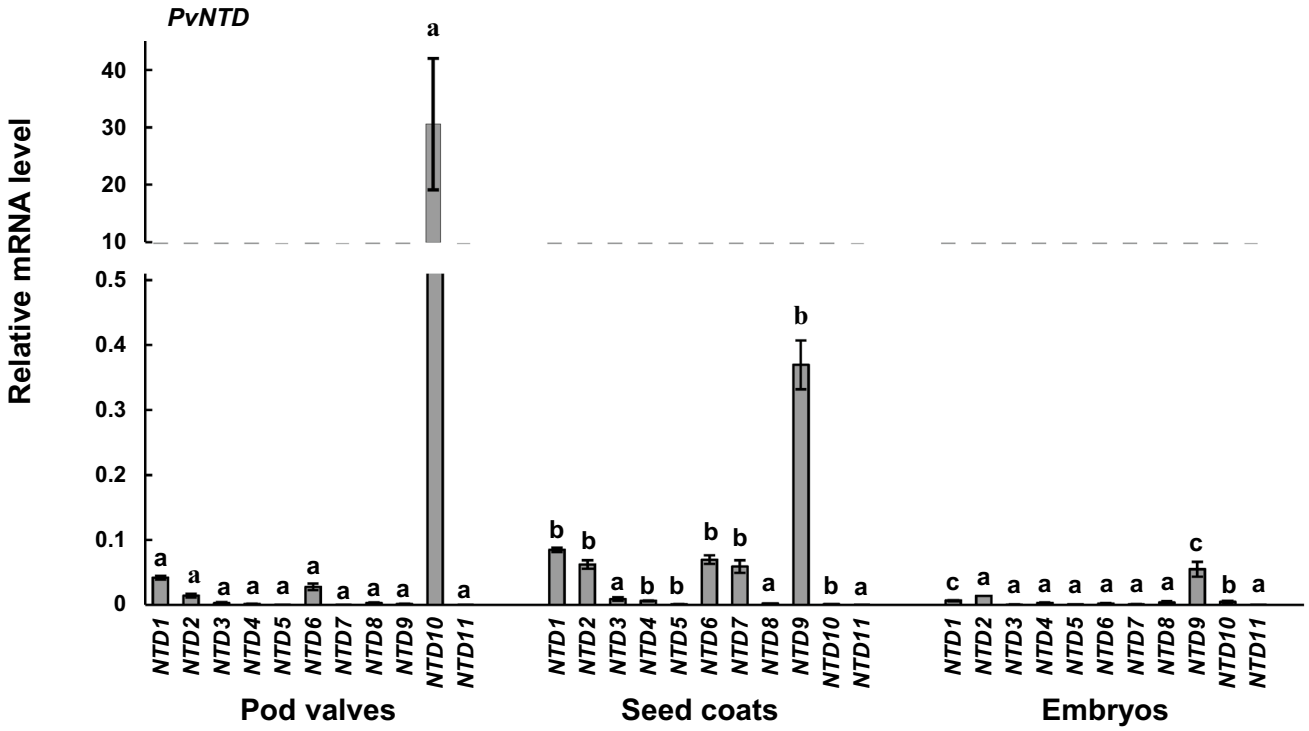

Fig. 6 Expression pattern of HAD family of putative nucleotidases (*PvNTD1* to *PvNTD11*) in valves, seed coats, and embryos of common bean pods at seed filling stage. Expression analysis was performed using qRT-PCR on total RNA samples extracted from valves, seed coats, and embryos of common bean pods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

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 (Fig. 6).

Nucleosidase activity and expression analysis of PvNSH1 and 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 (Fig. 7a). This activity has some similarities with nucleotidase activity, since it was detected in all the pods parts analyzed, although the nucleosidase activity in seed coats was the same that in valves (Fig. 7a).

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

Allantoinase activity and gene expression pattern

To discriminate if the purinic nucleobase are fully degraded in pods during the seed filling stage, the allantoinase activity was determined in the same extracts since allantoin is an intermediate in purinic nucleotide metabolism. Therefore, the determination of allantoinase activity would indicate the full degradation of purinic nucleotides. Allantoinase activity was mainly detected in seed coats with some activity in valves and negligible in embryos (Fig. 8a). The expression of *PvALN1* follows a similar pattern than the allantoinase activity, whereas the expression of *PvALN2* was negligible in any fruit part (Fig. 8b).

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 the

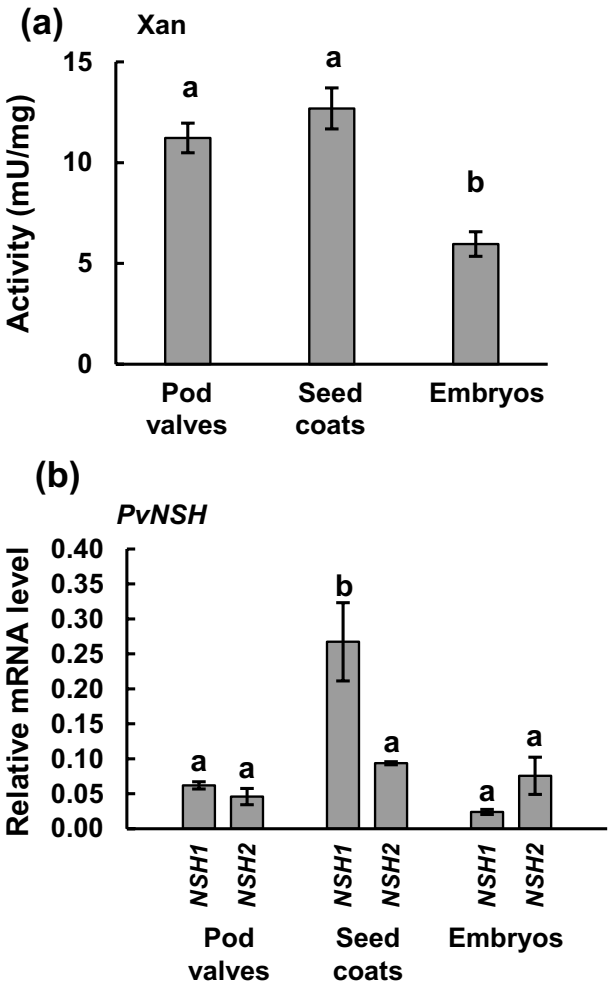

Fig. 7 Nucleosidase activity and expression pattern of two nucleosidase genes, PvNSH1 and PvNSH2, in pods of common bean. **a** Nucleosidase activity with xanthosine as substrate was determined in crude extracts obtained from valves, seed coats, and embryos of common bean pods at seed filling stage. Means ± SEs of three independent biological samples with two 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 analyzed using the $2^{-\Delta CT}$ method. Values are mean ± SE of three independent biological samples with two 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 ($p \leq 0.05$)

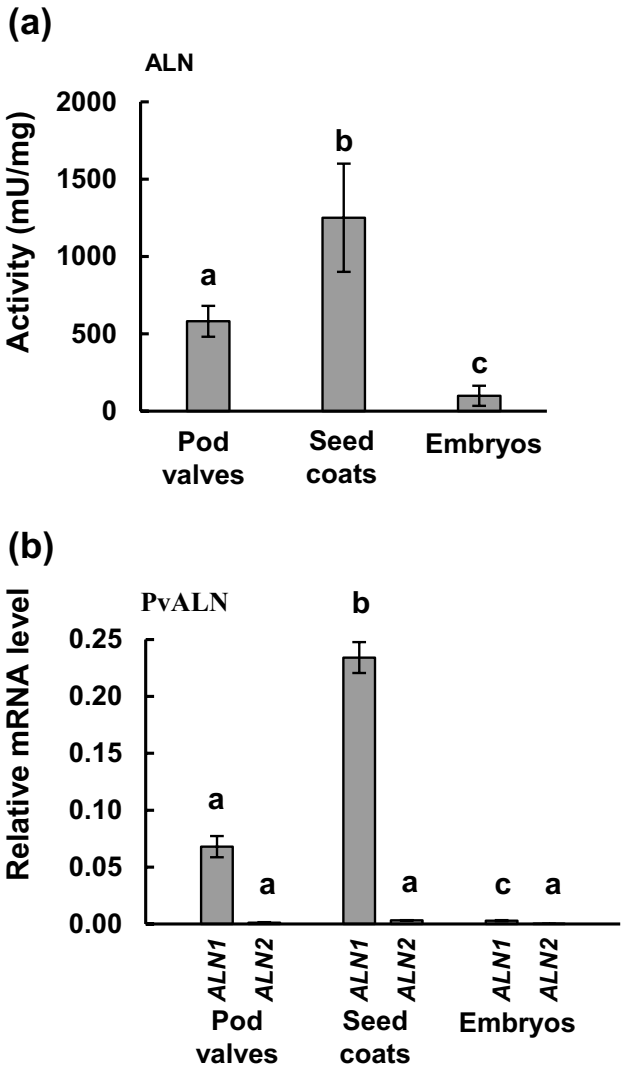

Fig. 8 Allantoinase activity and expression pattern of two allantoinase genes, *PvALN1* and *PvALN2*, in pods of common bean. **a** Allantoinase activity was assayed in crude extracts obtained from valves, seed coats, and embryos of common bean pods at the seed filling phase. Means ± SEs of three independent biological samples with two 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 analyzed using the $2^{-\Delta CT}$ method. Values are mean ± SE of three independent biological samples with two 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 ($p \leq 0.05$)

expression of the genes most highly expressed from each family analyzed in seed coats from fruits obtained from plants either grown 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 analyzed in seed coats were similar in both nitrogen regimes utilized (Fig. S2) without statistical differences.

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 analyze 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 analyzed 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 have been studied only in a few cases. In banana peel, nucleases are induced during ripening and over-ripening treatment (Ramirez-Sanchez et al. 2018), and in ethylene-treated immature cucumber fruit, induction of two bifunctional nucleases and four ribonucleases was 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 lacksf vascular connection with the embryo and is the tissue in which the phloem unloading occurs (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 indicates 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 analyzed the relative expression of genes families that encode the enzymatic activities analyzed 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 highlights 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 (Fig. 3), it 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 analyzed. 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 analyzed (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 in maintaining RNA levels in cells (Hillwig et al. 2011) 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 11 genes belonging 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 pyrimidic nucleosides and *PvNSH2* for purinic nucleosides (Delgado-García et al. 2021).

Allantoinase was expressed in seed coats and valves, *PvALN1* being 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 that 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 consumption and 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 (Cortes et al. 2022) and mainly for drought tolerance (Cortés et al. 2012, 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 analyzed, *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.

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Author contributions MDB, EDG, and IGdR performed the experiments, GGV and PP supervised the work, and PP and GGV wrote the manuscript.

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Data availability All data relevant to the study are included in the article.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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