Plant Genetic Resources: Characterization and Utilization

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Seed-stored transcript integrity as a molecular indicator of viability in conserved common bean germplasm

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Research Article

Cite this article: de Oliveira Silva Machado C *et al*. (2026) Seed-stored transcript integrity as a molecular indicator of viability in conserved common bean germplasm. *Plant Genetic Resources: Characterization and Utilization* **24**, 86–95. https://doi.org/10.1017/S1479262125100312

Received: 31 March 2025
Revised: 18 August 2025
Accepted: 8 September 2025
First published online: 13 October 2025

Keywords:
aging; conservation; germplasm; longevity; mRNA

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Abstract

Ensuring seed viability over decades is a central challenge in ex situ conservation of plant genetic resources. Standard germination tests, while effective, are destructive and fail to detect early molecular damage that precedes viability loss. RNA integrity has emerged as a promising biomarker due to the inherent vulnerability of mRNA to oxidative degradation in the dry state. In this study, we identified and validated seed-stored mRNAs in common bean (Phaseolus vulgaris), a major crop species extensively represented in global germplasm collections, and investigated transcript degradation patterns in seeds conserved for up to 30 years. Using a comparative genomics approach, we identified 107 P. vulgaris orthologs of long-lived rice mRNAs, many encoding proteins involved in RNA stabilization, oxidoreductase activity, and primary metabolism. Thirty transcripts were validated by RT-qPCR, and the integrity of nine was assessed using paired primers targeting 5′ and 3′ regions. Degradation followed a consistent 5′→3′ pattern, particularly in longer transcripts, and correlated strongly with germination power. Predictive performance varied across transcripts: *PSMA4*, *SMP1*, and *TRA2* consistently showed strong correlations with viability, whereas others were less informative. The ΔΔCq metric improved resolution by detecting degradation asymmetry. Samples included a genetically diverse panel of conserved, regenerated, and artificially aged accessions, enhancing applicability to real-world genebank conditions. Artificial aging intensified degradation signatures and mirrored patterns observed in naturally aged seeds. Altogether, our results indicate transcript integrity as a molecular biomarker for seed viability, supporting the development of robust molecular tools to inform decision-making and regeneration planning in long-term germplasm conservation.

Introduction

Plant genetic resources are fundamental to food security, sustainable agriculture, and biodiversity conservation. To ensure their long-term preservation and accessibility, more than 1,750 germplasm banks worldwide maintain extensive collections of plant genetic material (Fu 2017). Among the various conservation strategies, seed banking remains the most efficient and cost-effective approach for *ex situ* conservation, especially for species that produce orthodox seeds.

Orthodox seeds possess the remarkable ability to enter a quiescent physiological state, allowing them to survive desiccation and long-term storage at low temperatures without significant loss of viability (Walters 1998). When dried to approximately 5–6% moisture content and maintained under sub-zero conditions, these seeds can preserve their germination capacity for extended periods, making them well suited for *ex situ* conservation in genebanks. However, seed longevity is inherently limited, as aging processes gradually impair viability even under optimal storage conditions. These processes are associated with the accumulation of molecular damage over time, particularly due to the spontaneous generation of reactive oxygen species (ROS) during the dry state, which are not effectively neutralized in the absence of active metabolism (Walters *et al.,* 2005; Rajjou and Debeaujon 2008; Fleming *et al.,* 2018; Sano *et al.,* 2020).

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In this context, routine monitoring of seed viability is essential to guide regeneration decisions and prevent irreversible losses in germplasm collections. Germination tests remain the international standard for viability assessment, due to their simplicity and low cost. Nevertheless, they present several limitations: they are time-consuming, destructive, and insensitive to early molecular changes associated with seed aging (Walters *et al.,* 2005; Nagel and Börner 2010).

To overcome these limitations, molecular and biochemical biomarkers have been explored as tools to detect early deterioration (Walters 1998; Kurek *et al.,* 2019; Wang *et al.,* 2020), enabling faster and more proactive viability monitoring while minimizing seed usage, which is especially relevant for rare or limited samples (Hay and Whitehouse 2017; Wijnker *et al.,* 2024). These biomarkers often focus on macromolecules that are primary targets of ROS-induced oxidative damage, which accumulates progressively during storage in the dry, metabolically inactive state. This deterioration is often asymptomatic until viability is significantly compromised, posing a threat to genetic integrity in conservation systems (Rajjou and Debeaujon 2008).

RNA integrity has emerged as a particularly informative and sensitive indicator of seed aging. Unlike other macromolecules, RNA is structurally fragile due to its single-stranded conformation and inherent chemical instability, which renders it highly susceptible to oxidative damage. This structural vulnerability is particularly critical in seeds, where long-lived stored mRNAs perform essential regulatory and metabolic functions during the early stages of germination, before the reactivation of de novo transcription. The degradation of these transcripts impairs the translational capacity required for successful seedling establishment. Notably, mRNAs encoding stress-response proteins, translation factors, and key metabolic enzymes, such as heat shock proteins, eukaryotic release factors, and glycolytic enzymes are among the most conserved and functionally indispensable across species (Basbouss-Serhal *et al.,* 2017b; Sano *et al.,* 2020). Consistent with their essential role, evidence from multiple species has shown that, during long-term storage, the progressive degradation of these transcripts closely parallels declines in seed viability (Fleming *et al.,* 2018; Zhao *et al.,* 2020b; Walters *et al.,* 2005; Dantas *et al.,* 2025).

Two main approaches have been employed to assess transcript integrity: (i) measurement of global RNA quality using the RNA Integrity Number (RIN), and (ii) transcript-specific qRT-PCR assays that quantify degradation based on amplification efficiency at the 5′ and 3′ ends of selected transcripts. The difference in quantification cycle (ΔCq) values between these regions serves as a proxy for RNA fragmentation (Fleming *et al.,* 2018). Using this approach, degradation patterns have been detected in aged seeds of soybean, *Arabidopsis*, wheat, and canola, with longer transcripts and 5′-end amplicons showing greater susceptibility to deterioration. Importantly, loss of RNA integrity, often associated with preferential 5′ end degradation, can precede detectable reductions in germination performance, highlighting its potential as an early molecular marker of seed deterioration (Zhao *et al.,* 2020a, b). Moreover, the extent of transcript degradation has been significantly correlated with viability decline, reinforcing the value of RNA integrity metrics as predictors of physiological aging (Rajjou and Debeaujon 2008; Waterworth *et al.,* 2015; Nguyen *et al.*, 2015).

Building upon these findings, the present study investigates transcript integrity in dry-stored seeds of common bean (*Phaseolus vulgaris* L.), a species of major agronomic relevance and a priority in global germplasm conservation efforts.

Material and methods

Material

This study analyzed 20 seed samples of *Phaseolus vulgaris* including 11 long-term conserved accessions, two regenerated accessions (BRA11686-3 and BRA11682-2), one multiplied accession (BRA11853-9B), and six artificially aged samples of the 'Estilo' cultivar (Table 1). Embrapa Genetic Resources and Biotecnhology germplasm bank provided the 11 long-term preserved accessions that were used. The conserved accessions represent a genetically diverse subset of the Embrapa *P. vulgaris* germplasm collection, encompassing landraces, traditional varieties, and historical cultivars with broad geographical distribution. Their inclusion ensures that the transcript-based markers evaluated in this study are applicable to non-elite genetic resources, in line with the broader mission of genebanks to preserve diversity beyond modern breeding lines. Accessions were selected based on the availability of at least 1,500 seeds, documented variation in germination power (GP%) and RNA integrity, and the presence of duplicates in other germplasm repositories, including the Active Germplasm Bank of Embrapa Rice and Beans (Aparecida de Goiânia, GO, Brazil). The regenerated and multiplied accessions, representing the most recently produced seed lots, served as high-viability controls. Artificial aging treatments were applied to seeds of the cultivar 'Estilo' (initial GP = 85%) for 0, 48, 96, 120, 144, and 168 hours, following the protocol described by Marcos-Filho (1999). This treatment was implemented exclusively to simulate degradation under controlled conditions and to validate the transcript-based assay, irrespective of the cultivar's breeding status. Dantas *et al.* (2025) previously assessed germination power and RNA Integrity Number (RIN) values for all samples, including the aged, regenerated and multiplied lots. The same accession at 0 hours of artificial aging was used as the control, representing the initial (non-aged) state. To serve as a reference for long-term stored seeds, the regenerated material of accession BRA 11686-3 was used as the control for the samples obtained from the genebank.

To balance statistical robustness with material availability, two different bulk sizes were used for RNA extraction. Nine samples were analyzed using three independent bulks of 17 seeds each, while eleven samples were analyzed using a single bulk of 50 seeds. The triplicate bulks aimed to improve reproducibility and account for potential intra-sample variability, whereas the single larger bulk was intended to maximize representativeness while preserving material for future analyses. To evaluate whether bulk size affected RNA integrity assessment, a one-way ANOVA was performed to compare Cq values obtained from the two sampling strategies across all transcripts analyzed.

Methods

Identification of long-lived mRNAs in beans through comparative genomic analysis

The bean orthologs of rice genes encoding long-lived mRNAs were identified through a comparative genomic analysis based on orthologous groups, using OrthoFinder 3.0 (Emms and Kelly 2015). In rice, these long-lived mRNAs were characterized as RNA molecules stored in mature dry seeds, synthesized during the seed maturation process and transcribed during imbibition (Sano *et al.,* 2019).

https://doi.org/10.1017/S1479262125100312 Published online by Cambridge University Press

Table 1. Percentage of Germination Power (GP%) percentage and RNA Integrity Number (RIN) in seeds from 20 samples. Single or triplicate replicates were analyzed due to differences on seed number availability

Sample	GP% ± SD and RIN ± SD*	Number of biological replicates	Number of technical replicates in qPCR	Seeds analyzed per bulk
BRA 11686-3+ *(control)*	100.00 ± 0.00 and 9.16 ± 0.20	3	3	3 bulks × 17 seeds
BRA 11682-2+	100.00 ± 0.00 and 8.90 ± 0.10b	3	3	3 bulks × 17 seeds
BRA 11853-9B	99.00 ± 2.50 and 8.46 ± 0.05	3	3	3 bulks × 17 seeds
BRA 24297-4	97.50 ± 2.88 and 7.63 ± 0.06	1	3	1 bulk × 50 seeds
BRA 11853-9	97.50 ± 2.88 and 7.06 ± 0.05	1	3	1 bulk × 50 seeds
BRA 11723-4B	97.00 ± 2.88 and 7.37 ± 0.05	3	3	3 bulks × 17 seeds
BRA 12025-3	96.25 ± 2.50 and 7.86 ± 0.05	1	3	1 bulk × 50 seeds
BRA 13303-3	96.25 ± 2.50 and 7.84 ± 0.25	1	3	1 bulk × 50 seeds
BRA 11853-9B+	96.00 ± 8.94 and 9.66 ± 0.23	3	3	3 bulks × 17 seeds
BRA 11961-0	93.75 ± 6.29 and 7.88 ± 0.05	1	3	1 bulk × 50 seeds
BRA 12313-3	45.00 ± 4.08 and 6.10 ± 0.17	3	3	3 bulks × 17 seeds
BRA 11686-3	30.00 ± 10.80 and 3.53 ± 0.41	3	3	3 bulks × 17 seeds
BRA 11682-2	4.00 ± 2.50 and 3.60 ± 0.00	3	3	3 bulks × 17 seeds
BRA 11586-5	3.00 ± 2.88 and 5.27 ± 0.72	3	3	3 bulks × 17 seeds
Estilo, aged 0 h	87.00 ± 2.88a and 9.30 ± 0.04	1	3	1 bulk × 50 seeds
Estilo, aged 48 h	83.00 and 8.4*	1	3	1 bulk × 50 seeds
Estilo, aged 96 h	80.00 and 7.97**	1	3	1 bulk × 50 seeds
Estilo, aged 120 h	62.00 ± 5.77b and 7.72 ± 0.25**	1	3	1 bulk × 50 seeds
Estilo, aged 144 h	62.00 and 7.6***	1	3	1 bulk × 50 seeds
Estilo, aged 168 h	38.00 ± 2.88 and 7.32 ± 0.24***	1	3	1 bulk × 50 seeds

+Samples regenerated in 2023.
Germination power (GP) and RIN data were published in Dantas *et al.* (2025).
indicate significant differences ($p < 0.05$, **$p < 0.01$, **$p < 0.001$) between non-aged samples (0 h) and artificially aged samples, as determined by one-way ANOVA followed by Dunnett's test.

RNA integrity assessment in seeds

For each bean gene identified through comparative genomic analysis, the corresponding coding sequence (CDS) were retrieved from the *P. vulgaris* v2.1 genome annotation (Phytozome genome ID: 442) available in Phytozome (Goodstein *et al.,* 2012) via sequence similarity searches.

A set of 47 candidate genes was selected based on their reported involvement in rice seed germination, identified through comparative genomics analyses. From an initial list of 101 orthologs, genes were categorized by coding sequence (CDS) length: 31 genes were shorter than 1,200 bp, 50 ranged between 1,200 and 2,500 bp, and 26 exceeded 2,500 bp. To ensure size diversity in the assay, genes from each length category were selected for primer design. The objective was to identify at least 40 genes that could be successfully amplified from *P. vulgaris* genomic DNA under standardized PCR conditions.

To assess RNA integrity, specific primer pairs were designed to amplify regions near the 5′ and 3′ ends of each CDS using Primer3Plus (Untergasser *et al.,* 2012). Primers were selected based on stringent criteria: an amplicon size of 80–150 bp, primer length of 18–22 nucleotides, melting temperature between 57–63 °C, and GC content of 45–80%. Sequences prone to self-complementarity, hairpin formation, or secondary structures were excluded. Primer specificity was verified in silico using NCBI's Primer-BLAST.

Primer positions varied across CDS regions due to differences in optimal primer design, leading to variable coverage. Coverage was calculated using the formula: CDS Coverage (%) = (length between the 5′ and 3′ amplification primers/Total CDS length) × 100, where length between the 5′ and 3′ amplification sites corresponds to the distance from the 5′ end of the most upstream forward primer to the 3′ end of the most downstream reverse primer: total CDS length refers to the complete coding sequence of the transcript PCR and semi-quantitative RT-PCR analyses were performed to assess amplification efficiency and transcript detection across different conditions. Tables S3 and S4 provide details about each primer RNA extraction was performed using the PureLink™ Plant RNA Reagent (Thermo Fisher Scientific) and the Aurum™ Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad), following the spin protocol provided by the Bio-Rad kit manufacturer. DNA extraction was performed using the CTAB protocol described by Doyle and Doyle (1987), from hypocotyl tissue of the common bean cultivar Estilo. cDNA synthesis was carried out using the Promega GoScript™ Reverse Transcriptase kit. To confirm the presence of the target genes in the genome and their expression in seeds, two pairs of primers for each of the 47 CDS (Table S3) were tested by PCR on genomic DNA (gDNA) and RT-PCR on complementary DNA (cDNA) templates. PCR and RT-PCR reactions were conducted in a final volume of 25 µL, containing 17.8 µL of H_2O, 2.5 µL of 10 × *Taq* Polymerase buffer, 2.5 µL of dNTPs

https://doi.org/10.1017/S1479262125100312 Published online by Cambridge University Press

(2.5 mM), 0.5 µL of forward primer (10 mM), 0.5 µL of reverse primer (10 mM), 0.2 µL of *Taq* Polymerase (QuatroG), and 1 µL of gDNA or cDNA (20–25 ng/µL). The PCR conditions included an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds all primer pairs, and extension at 72°C for 40 seconds, with a final extension at 72°C for 2 minutes. PCR and semi-quantitative RT-PCR products were analyzed by electrophoresis in 1% (w/v) agarose gels. Representative amplification results for 10 primer pairs are shown in Figure S1.

Sampling strategy comparison for RNA integrity

The impact of sample size on RNA integrity assessment was evaluated by comparing the variance of Cq values from two sampling strategies: triplicate bulks of 17 seeds and a single bulk of 50 seeds. One-way ANOVA was applied separately for each transcript to test whether sampling strategy influenced Cq measurements. This analysis was performed to determine whether smaller, replicated bulks yield results consistent with those from a larger single bulk, without introducing systematic bias in RNA integrity estimation.

Assessment of RNA integrity in bean seeds using RIN and transcript-based ΔCq metrics

The RNA Integrity Number (RIN) for all extracted total RNA from bean seeds was previously measured by Dantas *et al.* (2025) using high-performance electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies) with the Agilent RNA 6000 Nano kit (Waldbronn, Germany) and the Plant RNA Nano assay (software version B.02.10, SI764), following the manufacturer's protocols. The RIN values were calculated using the Agilent 2100 software.

qRT-PCR reactions were performed on the QuantStudio 1 Real-Time PCR System (Thermo Fisher), as previously described by Morgante *et al.* (2013). The primer efficiency and melting curve can be seen in Table S4 and Figures S4 and S5.

Transcript degradation across samples was evaluated using methodologies based on Fleming *et al.* (2019) and Zhao *et al.* (2020a, b). Transcript integrity was estimated using ΔCq calculations:

- **ΔCq 3′**: Cq3′(sample) – Cq3′(control)
- **ΔCq 5′**: Cq5′(sample) – Cq5′(control)
- **ΔΔCq**: ΔCq5′ – ΔCq3′

Average ΔCq values and standard deviations were calculated from biological and technical replicates. The correlation between Cq, ΔCq and ΔΔCq values and the germination percentage (GP) of each sample was assessed using linear regression in Excel 2010.

Correlation analyses between Cq, ΔCq, and ΔΔCq values and germination percentage were performed using seed samples from 10 accessions, comprising both long-term stored materials and regenerated counterparts with contrasting germination capacities (2% to 100%; Table 1). The long-term stored samples included BRA 11853-9B (GP 99%), BRA 11853-9 (GP 97.5%), BRA 11723-4B (GP 97%), BRA 12313-3 (GP 45%), BRA 11686-3 (GP 30%), BRA 11682-2 (GP 4%), and BRA 11586-5 (GP 3%), while the regenerated samples were BRA 11686-3, BRA 11682-2, and BRA 11853-9B (all GP 100%). Cq values were obtained using cDNA synthesized from total RNA with 5′- and 3′-targeting primers (Figure 2).

Artificial intelligence tools

Figures 2, 3, S2 and S3 were prepared with the support of AI tools (OpenAI ChatGPT, version 5) for data visualization and graphical outputs. All statistical analyses and scientific interpretations were performed and validated by the authors.

Results

Putative seed-stored mRNAs in *P. vulgaris* were identified using a comparative genomics approach based on long-lived transcripts in dry rice embryos. Protein sequence similarity searches identified 107 putative orthologous genes in common bean (Table S1), with an average protein sequence identity of 81.06% ± 9.66%. These genes exhibited diverse coding sequence (CDS) lengths: 31 had CDSs < 1,200 bp, 50 ranged from 1,200 to 2,500 bp, and 26 exceeded 2,500 bp, with an overall average of 1,929 bp. Functional classification of the encoded proteins is detailed in Table S2.

Presence and transcriptional activity of these orthologous genes in *P. vulgaris* seeds were tested using primers designed for 47 representative CDSs. Forty genes showed successful amplification in genomic DNA, confirming their presence under the tested amplification conditions. The remaining seven genes are presumed to be present but were not amplified under these specific conditions, and 30 amplified cDNA, confirming transcript expression. Thus, 85% of the selected genes were present in the genome and 64% were transcriptionally active in dry seeds. Two primer pairs per gene targeting the 5′ and 3′ regions were tested using genomic DNA (gDNA) and complementary DNA (cDNA) from dry seeds (Table S3).

From the 30 expressed transcripts, nine were selected for integrity assessment based on high CDS coverage (mean = 87.06% ± 7.34%) and functional relevance. Their CDS lengths ranged from 459 bp (*LEA_2*) to 3,796 bp (*SF3B1*), with a mean of 1,491 ± 955 bp (Table S3). Primer positions and transcript coverage are shown in Figure 1.

The impact of sample size on RNA integrity measurements was assessed using one-way ANOVA, comparing Cq values from samples processed in triplicate bulks of 17 seeds versus a single bulk of 50 seeds. No significant differences were observed ($p > 0.05$) across all transcripts (Figure 3), indicating that both sampling strategies yielded consistent results without introducing systematic bias.

Across all samples, 5′ Cq values were consistently higher than those from the 3′ end, indicating preferential 5′-to-3′ degradation. Longer transcripts such as CBM (2,787 bp) and SF3B1 (3,786 bp) showed greater differences between regions, while shorter transcripts such as LEA_2 (459 bp) were less affected (Figure 2).

Artificially aged and long-term stored seeds exhibited distinct degradation profiles. Artificially aged seeds had significantly higher Cq values, particularly at the 5′ end, whereas long-term stored seeds showed more moderate increases. Statistical analysis confirmed significant differences between aging treatments for both transcript regions ($p \leq 0.01$; Figure S3). Interestingly, for some transcripts such as *PSMA4*.5′ and *SMP1*.5′, the artificially aged samples exhibited Cq values comparable to or even lower than those of the 0 h control (Estilo), which had not undergone artificial aging. The Estilo control, despite retaining full germination capacity, showed notable transcript-specific RNA degradation, with ΔCq values of +2.25 and +1.52 for *PSMA4* and *SMP1*, respectively. In contrast, other transcripts, including *CLPC* and *SF3B1*, showed minimal degradation, displaying nearly identical 5′ and 3′ Cq values (ΔCq ≈ 0). These results indicate that transcript-specific degradation had already begun prior to artificial aging, highlighting distinct RNA integrity patterns that precede measurable physiological deterioration.

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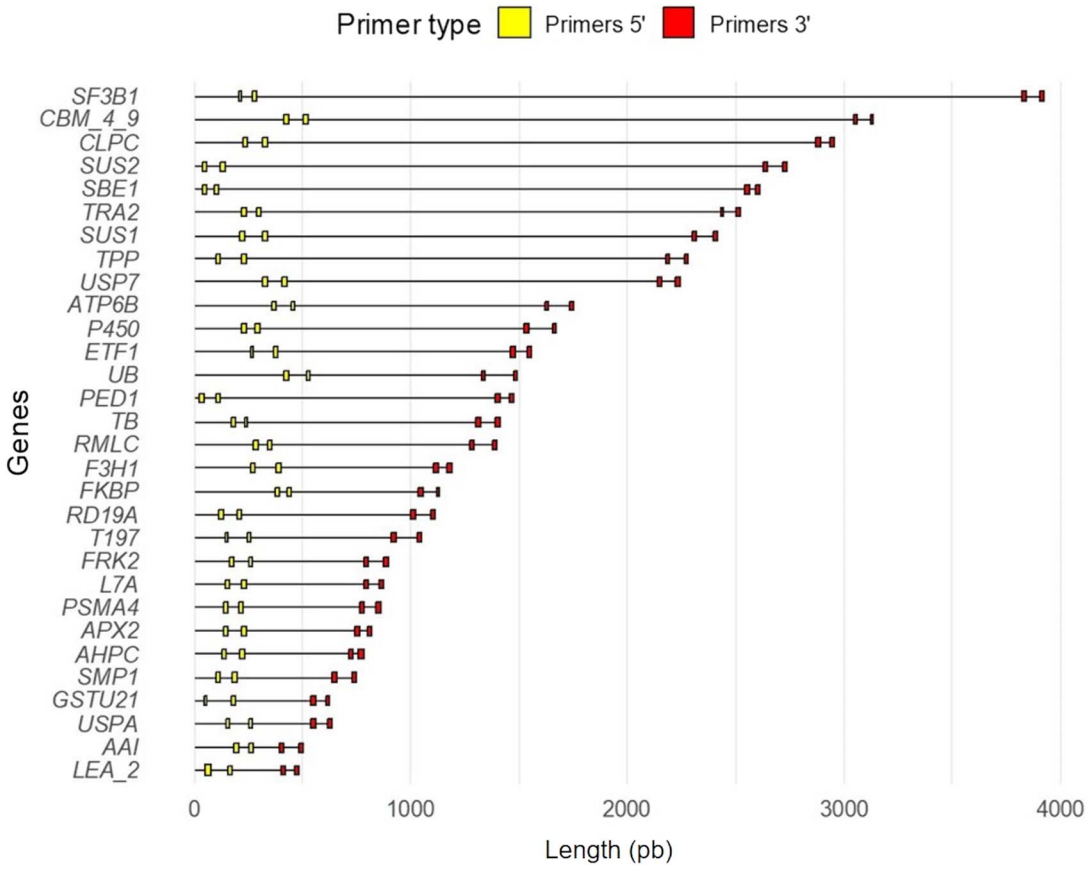


Figure 1. Distribution of primers along the analyzed CDS. The figure shows the positions of the two primer pairs used for cDNA amplification in 30 transcripts. The horizontal bars represent the length of the transcripts, extending from the 5' to the 3' ends. Primers positioned near the 3' end are shown in red (3' Primers), while those near the 5' end are shown in yellow (5' Primers). The variation in primer positions reflects design limitations due to the availability of suitable sequences for amplification along the coding sequence (CDS). The nine CDS analyzed in this study was *LEA_2* (Phvul.010G104800.1), *SMP1* (Phvul.007G057900.1), *PSMA4* (Phvul.008G093300.1), *PED1* (Phvul.007G236600.1), *ATP6B* (Phvul.007G018000.2), *TRA2* (Phvul.001G135200.1), *CLPC* (Phvul.009G162100.1), *CBM_4_9* (Phvul.001G032300.1) and *SF3B1* (Phvul.006G175600.1).

Associations between transcript integrity and seed viability were explored by grouping samples according to germination percentage (GP): high (GP $\geq$ 93%, n = 9), medium (GP = 45% and 30%, n = 2), and low (GP = 4% and 3%, n = 2). Transcripts such as *LEA_2* exhibited low intra-group variation in Cq values among high-GP samples, indicating consistent transcript stability. In contrast, *SMP1.5′* and *PSMA4.5′* showed higher Cq values in low-GP samples when compared to high-GP samples (Figure 3), suggesting increased 5′ degradation in seeds with reduced viability. Although these trends were visually evident, no formal statistical analysis was performed due to the limited number of samples in the medium and low-GP groups. Therefore, the interpretation of group differences remains descriptive and exploratory. Some variability was also observed within the high-GP group, suggesting that low-level degradation may occur even in viable seeds.

To evaluate the predictive performance of RNA integrity metrics, correlation analyses were performed between germination power (GP) and Cq, ΔCq, and ΔΔCq values for nine transcripts (Table 2). The results revealed transcript-specific differences in predictive capacity, with several 5′ regions showing strong and statistically significant associations with GP. Notably, *PSMA4.5′* (R^2 = 0.9178, p = 1.29E-05), *SMP1.5′* (R^2 = 0.8832, p = 5.35E-05), and *TRA2.5′* (R^2 = 0.91, p = 1.26E-05) consistently exhibited the highest predictive values across all three models ($p < 0.0001$), indicating their high sensitivity to RNA degradation. In contrast, transcripts such as *CBM.5′* and *SF3B1.5′* showed weak correlations in the Cq and ΔCq models ($R^2 < 0.50$), although their ΔΔCq values demonstrated improved association with GP. The *LEA_2.5′* transcript, in particular, showed moderate correlation in the Cq model (R^2 = 0.65) but exceptionally high predictive power in the ΔΔCq model (R^2 = 0.96, p = 3.14E-06), supporting its potential as a stable internal reference. These findings, summarized in Table 2 and illustrated in Figures 4I and 4J demonstrate that the diagnostic utility of transcript-based markers depends on both the identity of the transcript and the analytical model applied, with the ΔΔCq approach consistently enhancing sensitivity to degradation.

Discussion

The comparative evaluation of bulk sizes for RNA extraction demonstrated that both sampling strategies yielded consistent transcript integrity assessments. This supports methodological flexibility in seed conservation and molecular diagnostics, particularly when seed availability is limited. Similar observations were reported by Tetreault *et al.* (2022), who found that transcript integrity metrics in soybean seed were sensitive and consistent even when using reduced sample sizes. Other studies have also emphasized that RNA integrity declines predictably with seed aging (Fleming *et al.*, 2018; Kranner *et al.*, 2010), but explicit investigations of how bulk size affects transcript-based diagnostics

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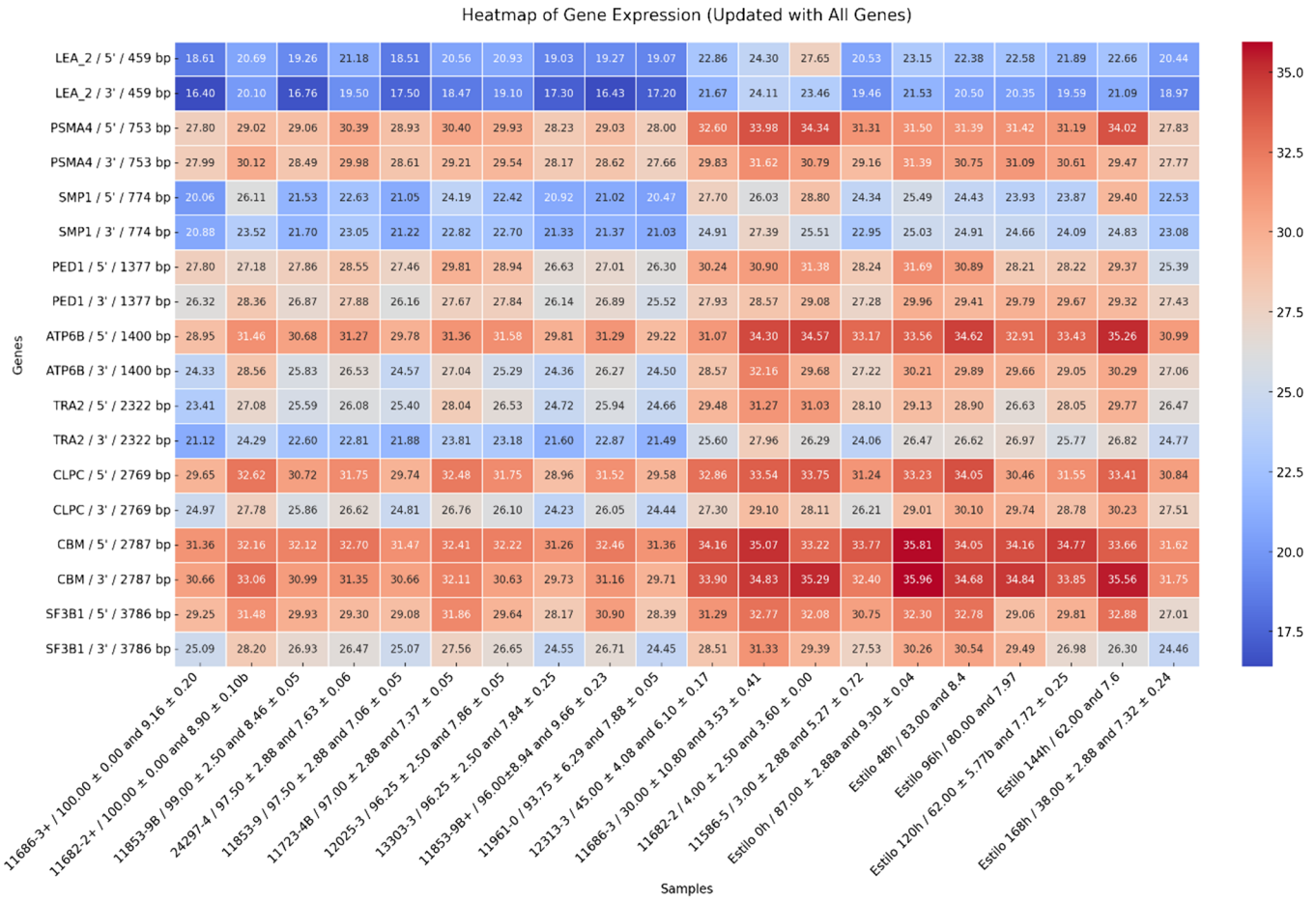


Figure 2. Heatmap presentings the Cq (Cycle Quantification) values for gene expression across different samples represented on the X-axis. Samples are identified by their codes and associated characteristics, including germination power (GP) and RNA Integrity Number (RIN) values. The evaluated CDS are represented on the Y-axis listed, accompanied by their primers (5' or 3') and the length of the amplified fragments in base pairs (bp). Cell values correspond to the Cq of each transcript in each sample. The coloring uses the 'coolwarm' scale, where dark blue represents low Cq values (higher gene expression), dark red indicates high Cq values (lower gene expression), and white reflects intermediate values.

remain scarce. These findings collectively support the operational feasibility of using smaller seed numbers in RNA-based monitoring of seed viability, which is particularly relevant for rare or limited-access germplasm.

The identification of 107 orthologous genes in *P. vulgaris*, based on seed-stored mRNAs initially described in rice, suggests that a core set of transcripts involved in early germination may be conserved across distantly related angiosperms. Functional classification revealed enrichment in genes related to processes known to be activated upon imbibition such as oxidative stress responses, RNA stabilization, carbohydrate metabolism, and translation initiation.

Validation of gene presence and expression confirmed that a majority of the orthologous genes are both conserved in the genome and stored as stable transcripts in dry seeds, even after long-term storage. The detection of transcripts such as *LEA, SF3B1, ATP6B*, and *ETF1* (a putative *eRF1* homolog) supports the model in which stored mRNAs act as a functional reserve, sustaining protein synthesis after imbibition and prior to the onset of de novo transcription. This finding reinforces the biological relevance of transcript-based viability markers, particularly for genes involved in processes known to be critical for early germination such as desiccation tolerance, mRNA splicing, energy metabolism, and translation initiation. In contrast, the absence of transcripts related to regulatory functions, such as MYB transcription factors, indicates that their activation may depend on imbibition or environmental cues, suggesting a separation between preloaded metabolic transcripts and stress-responsive regulatory programs. These results are consistent with previous transcriptome studies in *Arabidopsis*, rice, and soybean, which have shown that only a subset of mRNAs contributes functionally to early post-imbibition processes (Basbouss-Serhal *et al.*, 2017b; Fleming *et al.*, 2018; Sano *et al.*, 2020). Collectively, this supports the use of specific seed-stored transcripts as reliable molecular indicators of physiological status and is consistent with the hypothesis that translational preparedness via seed-stored transcripts contributes to seed longevity across species.

The directional degradation gradient observed from the 5′ to the 3′ end of transcripts, particularly pronounced in longer mRNAs such as *CBM* and *SF3B1,* is consistent with oxidative RNA damage mechanisms previously proposed for dry seeds. These 5′-biased decays may reflect structural vulnerabilities possibly including untranslated regions (UTRs), although our qPCR targets were restricted to coding sequences (CDS). Artificial aging treatments intensified this directional pattern, reinforcing the sensitivity of the assay to molecular deterioration prior to visible becomes apparent.

https://doi.org/10.1017/S1479262125100312 Published online by Cambridge University Press

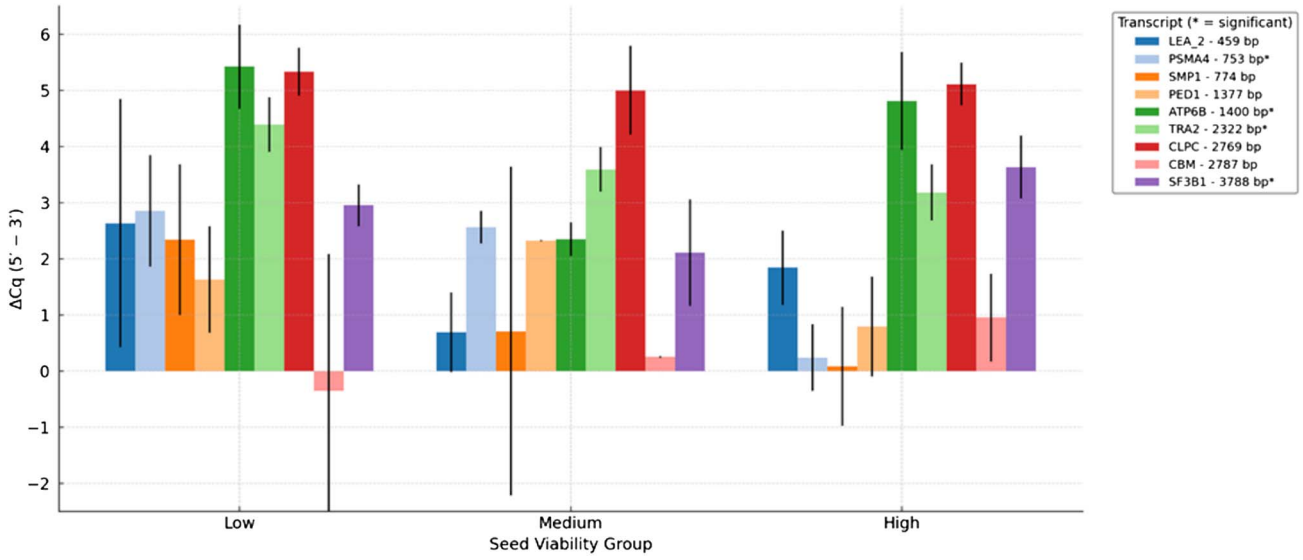


Figure 3. Mean ΔCq values for the 5′ regions of selected transcripts in seed samples grouped by germination percentage (GP): high (≥93%), medium (30–45%), and low (≤4%). Higher ΔCq values indicate greater 5′ degradation relative to 3′ ends. Transcripts such as *SMP1.5′* and *PSMA4.5′* showed increased ΔCq in low-GP samples, while *LEA_2* was stable across high-GP samples. Bars represent biological means ± standard deviation. Asterisks (*) indicate statistically significant differences between groups based on one-way ANOVA ($p < 0.05$).

These findings are consistent with models of regulated mRNA decay during seed transitions (Basbouss-Serhal *et al.,* 2017a) and extend current approaches by demonstrating that targeted qPCR using short amplicons positioned at opposite transcript ends (5′ and 3′) can resolve degradation asymmetry with high sensitivity. This refinement, in contrast to previous approaches based on one short and one long fragment minimizes amplification bias and enables accurate, region-specific profiling of transcript integrity at a transcript-specific level. Notably, the use of primers targeting both 5′ and 3′ of the coding sequence (CDS) may further enhance the predictive value of this assay, as some transcripts may retain partial coding sequence (CDS) integrity even when degradation has already compromised the upstream 5′ UTR. This refinement could improve the correlation between RNA integrity and germination performance.

The varying levels of RNA integrity observed in the Estilo 0 h sample, which served as a baseline control, highlight early molecular alterations in seeds prior to detectable viability loss. ΔCq values observed for *PSMA4* and *SMP1* were elevated, in line with oxidative RNA damage typically reported during seed (Fleming *et al.*, 2018; Walters *et al.*, 2005). Conversely, the minimal degradation observed for *CLPC* and *SF3B1* may reflect intrinsic stability due to transcript-specific structural protection or reduced susceptibility to oxidation in the dry state, as suggested by studies reporting selective RNA preservation in dry seeds (Bazin *et al.,* 2011; Nelson *et al.,* 2017). Thus, the unexpectedly high Cq values in certain control transcripts do not contradict the results but rather emphasize the complexity inherent to interpreting RNA integrity metrics. These findings underscore the necessity of employing multiple positional markers, careful temporal control, and caution when inferring RNA degradation solely from isolated transcript measurements, particularly in seeds exhibiting high physiological resilience.

Notably, for a few transcripts, the Cq values observed in the Estilo cultivar at 0 h were unexpectedly high and, in some cases, comparable to those of aged samples. This pattern may reflect transcript-specific differences in stability, residual oxidative stress affecting transcript abundance, or even technical variation during RNA extraction and cDNA synthesis. It is also possible that localized degradation outside the amplified region influenced Cq measurements without fully compromising transcript integrity. Previous studies have shown that long-lived mRNAs can exhibit non-linear degradation dynamics, especially for transcripts associated with desiccation tolerance or stress adaptation. Therefore, caution is warranted when interpreting absolute Cq values from individual transcripts. These observations underscore the importance of using multiple positional markers and composite metrics, such as ΔCq and ΔΔCq, to more accurately assess RNA integrity across samples with differing physiological conditions.

Among the transcripts analyzed, *LEA_2* stood out for its stable expression and low ΔCq variation within high-GP samples, supporting their suitability as reference transcripts for RNA integrity normalization in viability studies. This is consistent with the well-documented role of LEA proteins in protecting macromolecular structures under desiccation stress (Maia *et al.,* 2021). In contrast, transcripts such as *SMP1* and *PSMA4* were markedly degraded in low-GP samples, suggesting their potential as sensitive indicators of viability loss (Nguyen *et al.*, 2015). Conversely, *CBM.5′* and *SF3B1.5′* showed weaker or inconsistent associations with germination power, suggesting that the degradation of certain mRNAs may not reflect viability loss directly. Similar patterns have been reported in soybean and *Arabidopsis*, where some seed-stored transcripts deteriorate over time without compromising germination, possibly due to structural stability or limited functional relevance during early germination (Fleming *et al.,* 2018; Sano *et al.,* 2020). Additional transcripts such as *ATP6B.5′* and *CLPC.5′* also exhibited notable predictive potential, highlighting the importance of transcript selection for molecular viability diagnostics.

The presence of some intra-group variation among high-GP samples, particularly in longer transcripts, suggests background levels of RNA degradation even in physiologically viable seeds. This phenomenon has been observed in other species and may reflect transcript-specific susceptibility to damage, seed moisture content,

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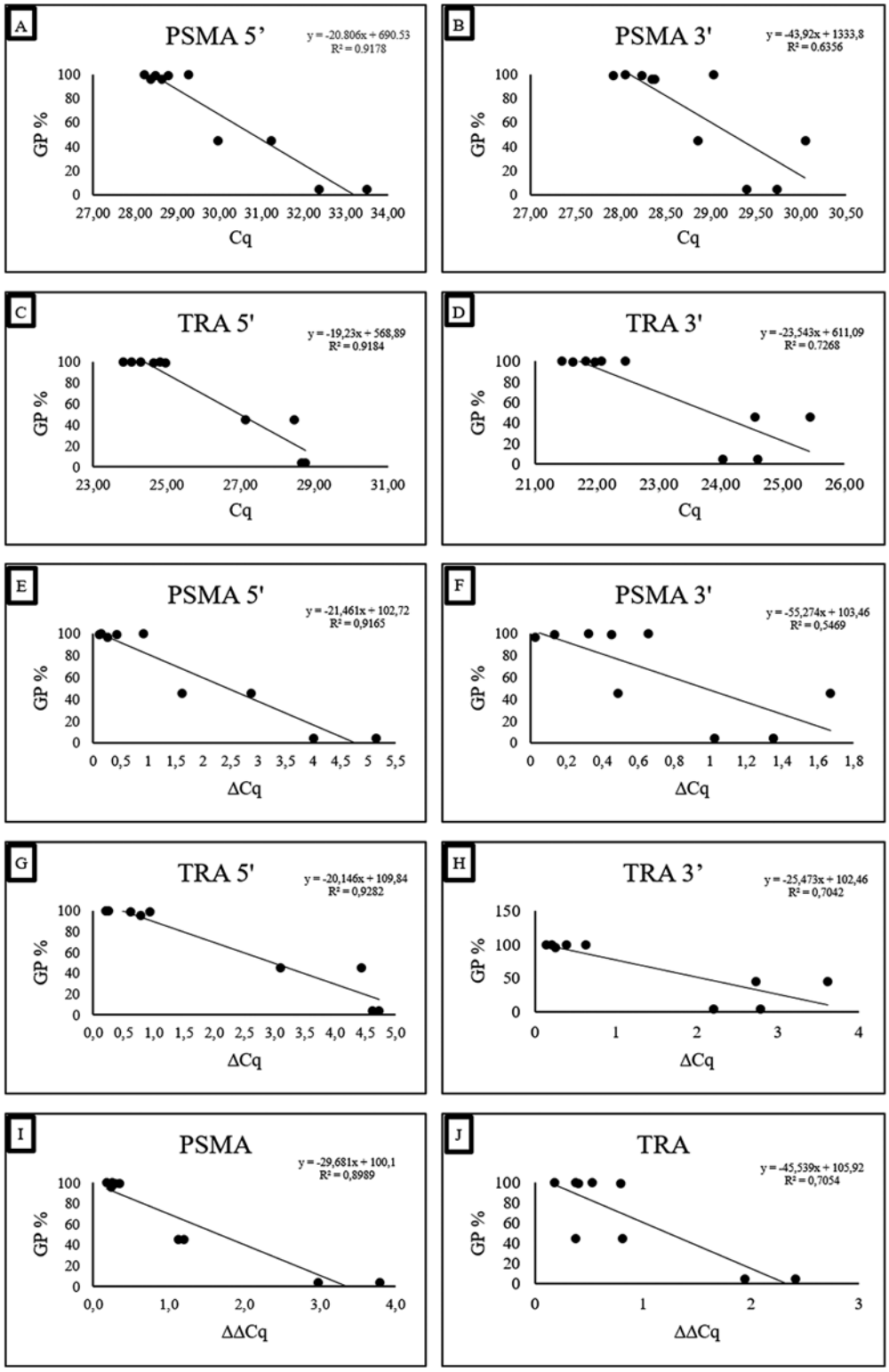


Figure 4. Correlation analyses between Cq values and germination percentage from 10 accessions. The long-term stored samples included: BRA 11853-9B (GP 99%), BRA 11853-9 (GP 97.5%), BRA 11723-4B (GP 97%), BRA 12313-3 (GP 45%), BRA 11686-3 (GP 30%), BRA 11682-2 (GP 4%), and BRA 11586-5 (GP 3%). The regenerated samples were: BRA 11686-3 (GP 100%), BRA 11682-2 (GP 100%), and BRA 11853-9B (GP 100%). Nine genes were analyzed via qPCR, with two genes presented above. The average Cq values were calculated from three biological and three technical replicates. The ΔCq and $\Delta\Delta$Cq was calculated as follows: ΔCq 5' = Cq 5' sample - Cq 5' control, and ΔCq 3' = Cq 3' sample - Cq 3' control, $\Delta\Delta$Cq = ΔCq 5' - ΔCq 3'. (A) Cq values of *PSMA.5'* in relation to GP. (B) Cq values of *PSMA.3'* in relation to GP. (C) Cq values of *TRA.5'* gene in relation to GP. (D) Cq values of *TRA.3'* in relation to GP. (E) ΔCq values of *PSMA.5'* transcript across seed samples and correlation with GP. (F) ΔCq values of *PSMA.3'* transcript across seed samples and correlation with GP. (G) ΔCq values of *TRA.5'* CDS across seed samples and correlation with GP. (H) ΔCq values of *TRA.3'* CDS across seed samples and correlation with GP. (I) $\Delta\Delta$Cq values of *PSMA* gene across seed samples and their correlation with GP. (J) $\Delta\Delta$Cq values of *TRA* gene across seed samples and their correlation with GP. Both linear regressions were generated using Microsoft Excel 2010, with $P < 0.005$, indicating significant correlations between Cq, ΔCq and $\Delta\Delta$Cq values and GP.

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Table 2. Correlation between Cq, ΔCq, and ΔΔCq values with germination power (GP) for different CDS at the 5′ and 3′ ends. Results were obtained for three models: Cq x GP, ΔCq x GP, and ΔΔCq x GP, for each CDS and end. Correlation analyses between Cq, ΔCq, and ΔΔCq values and germination percentage were performed using seed samples from 10 accessions. The long-term stored samples included: BRA 11853-9B (GP 99%), BRA 11853-9 (GP 97.5%), BRA 11723-4B (GP 97%), BRA 12313-3 (GP 45%), BRA 11686-3 (GP 30%), BRA 11682-2 (GP 4%), and BRA 11586-5 (GP 3%). The regenerated samples were: BRA 11686-3 (GP 100%), BRA 11682-2 (GP 100%), and BRA 11853-9B (GP 100%). P values < 0.05 indicate statistically significant correlations

CDS	R^2 Cq x GP/p-value	R^2 ΔCq x GP/p-value	R^2 ΔΔCq x GP/p-value
LEA_2.5′	0.65/4.81E-03	0.63/0.01	*0.96/3.14E-06*
LEA_2.3′	0.86/9.07E-05	0.86/2.99E-04	
PSMA4.5′	0.91/1.29E-05	0.91/5.07E-05	*0.90/9.97E-05*
PSMA4.3′	0.63/5.74E-03	0.55/0.02	
SMP1.5′	0.88/5.34E-05	0.90/1.32E-04	*0.95/6.51E-06*
SMP1.3'	0.64/6.0E-03	0.61/0.01	
PED1.5′	0.73/1.83E-03	0.73/3.30E-03	*0.63/9.78E-03*
PED1.3'	0.60/9.14E-03	0.56/0.01	
ATP6B.5′	0.76/9.48E-03	0.75/2.49E-03	*0.76/2.32E-03*
ATP6B.3'	0.67/4.01E-03	0.64/8.85E-03	
TRA2.5′	0.91/1.26E-05	0.93/3.08E-05	*0.70/4.60E-03*
TRA2.3'	0.73/1.72E-03	0.71/4.42E-03	
CLPC.5′	0.91/1.41E-05	0.91/5.26E-05	*0.57/0.01*
CLPC.3′	0.58/1.14E-02	0.57/0.01	
CBM.5′	0.16/0.23	0.01/0.72	*0.73/3.49E-03*
CBM.3′	0.71/2.48E-03	0.70/4.78E-03	
SF3B1.5′	0.49/0.02	0.48/0.03	*0.73/3.36E-03*
SF3B1.3′	0.76/8.93E-04	0.76/2.25E-03	

or storage time (Fleming *et al.,* 2018; Zhao *et al.,* 2020). Although such degradation may not compromise germination under optimal conditions, it may contribute to residual variance in predictive models and should be taken into account when defining molecular thresholds for viability prediction.

The variation in predictive capacity among transcripts underscores the importance of transcript selection for RNA-based viability diagnostics. While ΔCq and ΔΔCq approaches generally offer superior sensitivity compared to absolute Cq values, their reliability is highly dependent on transcript-specific degradation kinetics. Transcripts with moderate length, high expression, and biological relevance to early germination are more informative (Basbouss-Serhal *et al.,* 2017a; Sano *et al.,* 2020). Overall, these findings support the use of transcript integrity measurements, particularly at the 5′ region, as a promising molecular indicator of seed viability and as a potential tool to inform decision-making for improving decision-making in germplasm conservation programs.

Conclusions and perspectives

RNA integrity represents a quantifiable molecular parameter for detecting seed deterioration, particularly through the progressive loss of 5′ transcript ends. Although the number of samples with medium and low germination power (GP) was limited, reflecting the scarcity of long-term stored accessions with reduced viability. These findings support the potential of transcript integrity as a molecular marker of seed aging. Notably, seeds with comparable GP values displayed different degrees of RNA degradation, indicating that transcript-based analyses can detect early molecular deterioration that precedes visible loss of viability. Furthermore, not all transcripts exhibited the same diagnostic value. While several 5′ regions, including *SMP1.5′*, *PSMA4.5′*, and *TRA2.5′*, showed strong and consistent associations with GP across all models, others such as *CBM.5′* and *SF3B1.5′* demonstrated weaker or model-dependent correlations. The ΔΔCq metric enhanced resolution by capturing asymmetries in degradation between transcript ends, underscoring the relevance of both transcript selection and analytical model design for RNA-based diagnostics. The observed association between 5′-to-3′ degradation gradients and germination performance support the diagnostic potential of integrity-based metrics for refining seed quality assessment. While this study demonstrates the applicability of qPCR-based integrity assays in *Phaseolus vulgaris*, further evaluation across additional species and seed lots is needed to confirm the selected markers are broadly applicable. Integrating transcriptome-wide analyses, such as RNA-Seq, may enhance marker resolution and reveal transcript-specific susceptibility to degradation. Additionally, elucidating how storage conditions influence RNA stability over time will contribute to the refinement of molecular viability monitoring frameworks. Overall, coupling RNA integrity assays with conventional germination tests provides a biologically relevant and minimally invasive approach to improve seed conservation strategies and guide regeneration decisions in genebank management.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S1479262125100312.

Acknowledgements. We sincerely thank the Federal District Research Support Foundation (FAP-DF, Brasília, Brazil, process number FAP 00193-00000928-71) for funding this research and supporting scholarships for Cristielly Machado, Tayara Nunes, Rutiane Moreira and Thyfani Purcena. We also thank Thyfani Purcena for her significant contributions to the bioinformatics analyses, Dr. Paula Torga (Embrapa Rice and Bean) for her valuable assistance in the regeneration and multiplication of accessions and Alisson Dantas for the germination and artificial aging assays. The authors used AI tools (ChatGPT, version GPT-5; OpenAI) to assist with the editing and organization of the manuscript. All intellectual content and interpretations are solely the responsibility of the authors.

Competing interests. The authors have no relevant financial or non-financial interests to disclose.

Author contributions. All authors contributed to the conception and design of the study, as well as to the analysis and interpretation of the results. Marcos Gimenes served as the lead researcher and study coordinator and securing funding. Marcos also designed primers for the analyses. Marília Pappas and Tayara Nunes optimized the RNA extraction protocol, performed RNA extractions, analyzed RNA quality using the BioAnalyzer. Cristielly Machado, Mario Saraiva and Ana C. Brasileiro, and Mario Saraiva tested the primers on gDNA and cDNA, conducted qPCR, and carried out gene expression analyses, including linear regression between germination power (GP) and Cq values. Priscila Grynberg and Rutiane Costa identified orthologs from rice to beans. Solange José, Antonieta Salomão, Juliano Pádua, Rosana Brondani were responsible for the regeneration and multiplication of accessions. All authors contributed to writing and revising the manuscript, providing critical insights to improve its quality.

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