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The identification of suitable internal reference genes in quinoa seeds subjected to abscisic acid and gibberellin treatment

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ABSTRACT: Quinoa has been recognized as the sole "comprehensive nutritional crop"; however, it is susceptible to pre-harvest sprouting (PHS). While quantitative reverse transcription polymerase chain reaction (RT-qPCR) has been extensively employed for gene expression level detection, the selection of suitable reference genes is imperative to ensure precise gene expression quantification across diverse conditions. This study aims to identify stable reference genes in quinoa seeds under ABA and GA, in order to provide a basis for subsequent research on PHS. Seeds were subjected to different concentrations of ABA and GA (10 µM, 50 µM, 100 µM, and 200 µM). The most suitable treatment concentration was determined based on seed viability. Here, *MON1, GAPDH, EIF3, EF1α, ACT, TUB1,* and *TUB6* were selected as candidate genes. The suitability of these reference genes under different conditions was assessed using various methods including Ct values, geNorm, NormFinder, BestKeeper, Delta Ct, and RefFinder. Based on the results obtained from the hormone experiments, it was observed that the application of 100 µM ABA and 200 µM GA yielded the most advantageous outcomes. Additionally, the most appropriate reference genes for different treatments are *ACT* and *TUB1* (H_2O treatment), *EIF3* and *MON1* (ABA, GA treatment and also for the combined data set of the three groups). However, *GAPDH* exhibited the least stability across all treatments. In summary, *ACT* is recommended as the reference gene for natural quinoa germination, while *EIF3* and *MON1* should be used for ABA and GA treatments.

Index terms: ABA, *Chenopodium quinoa* Willd.; GA, reference gene, RT-qPCR.

RESUMO: A quinoa foi reconhecida como a única "cultura nutricional abrangente"; no entanto, é suscetível à germinação na pré-colheita (BHS). Embora a reação em cadeia da polimerase com transcrição reversa quantitativa (RT-qPCR) tenha sido amplamente empregada para detecção do nível de expressão gênica, a seleção de genes de referência adequados é essencial para garantir a quantificação precisa da expressão gênica em diversas condições. Este estudo tem como objetivo identificar genes de referência estáveis em sementes de quinoa tratadas com ABA e GA, a fim de fornecer uma base para pesquisas subsequentes em BPC. As sementes foram submetidas a diferentes concentrações de ABA e GA (10 µM, 50 µM, 100 µM e 200 µM). A concentração de tratamento mais adequada foi determinada com base na viabilidade das sementes. *MON1, GAPDH, EIF3, EF1α, ACT, TUB1* e *TUB6* foram selecionados como genes candidatos. A adequação destes genes de referência sob diferentes condições foi avaliada utilizando vários métodos, incluindo valores Ct, geNorm, NormFinder, BestKeeper, Delta Ct e RefFinder. Com base nos resultados obtidos nos experimentos com hormônios, observou-se que a aplicação de 100 µM de ABA e 200 µM de GA produziu os resultados mais vantajosos. Além disso, os genes de referência mais apropriados para diferentes tratamentos são *ACT* e *TUB1* (tratamento com H_2O), *EIF3* e *MON1* (tratamento com ABA, GA e para o conjunto de dados combinados dos três grupos). No entanto, o GAPDH exibiu a menor estabilidade em todos os tratamentos. Em resumo, o ACT é recomendado como gene de referência para a germinação natural da quinoa, enquanto o *EIF3* e o *MON1* devem ser utilizados para os tratamentos com ABA e GA.

Termos para indexação: ABA, *Chenopodium quinoa* Willd.; GA, gene de referência, RT-qPCR.

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INTRODUCTION

Chenopodium quinoa Willd., a member of the Amaranthaceae family indigenous to South America, possesses a rich composition of proteins, vitamins, minerals, dietary fiber, plant sterols, phenolic compounds, and essential amino acids that align with human nutritional requirements (Dakhili et al., 2019). Quinoa is susceptible to pre-harvest sprouting (PHS), resulting in substantial losses. The global economic losses attributed to PHS are estimated to be reach one billion dollars annually (Tai et al., 2021). Breaking dormancy at an inappropriate time may affect plant morphogenesis or lead to the occurrence of PHS (Kashiwakura et al., 2016). Dormancy and germination processes are influenced by a multitude of factors, including temperature, air humidity, soil moisture content, exogenous chemicals, genetic factors, reactive oxygen species levels, seed maturity, and hormone levels (Barrero et al. 2020; Wang et al. 2020; Sohn et al. 2021; Pelissari et al. 2022; Rabieyan et al. 2022; Pinto et al. 2023). Among these factors, the hormones Abscisic acid (ABA) and Gibberellin (GA) exert a particularly significant impact (Barrero et al., 2020; Wang, et al., 2020; Sohn et al., 2021; Pelissari et al., 2022; Pinto et al., 2023; Rabieyan et al., 2022). The synthesis and metabolism of ABA and GA involve various multiple enzyme-catalyzed processes. Enzymes such as zeaxanthin epoxidase, 9-cis-epoxycarotenoid dioxygenase, and ABA-aldehyde oxidase participate in ABA synthesis, while ABA 8'-hydroxylase (ABA8ox) is responsible for the degradation of ABA into Phaseic acid (Seo and Koshiba 2002; Minami et al. 2018; Perreau et al. 2020; Wang et al. 2021; Jia et al. 2022). GA is produced through a sequence of enzymatic reactions involving precursor substances. These reactions are catalyzed by GA 20-oxidases and GA 3-oxidases, resulting in the formation of biologically active GA forms. Conversely, GA can be deactivated by the activity of GA2 oxidases (GA2ox) (Lor and Olszewski, 2015).

Examining the expression levels of genes associated with PHS is an imperative approach to facilitate the resolution of PHS. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) has been extensively utilized in the domains of medical and scientific research, for the purpose of detecting gene expression levels and investigating gene transcription, regulation, and validation (Ma et al., 2020; Wu et al., 2021).In order to ensure the utmost reliability of experimental outcomes, it is imperative to implement stringent control measures for crucial experimental variables, including sample quality control, experimental design, statistical analysis, and the selection and validation of reference genes, owing to the heightened sensitivity and specificity of the methodology (Škiljaica et al., 2022). In the context of RT-qPCR, the accurate acquisition of results heavily relies on the meticulous selection of suitable reference genes for data normalization (Deng et al., 2016).

In theory, reference genes are characterized as genes whose expression remains unaltered by variations in tissue types, experimental conditions, or environmental factors (Zhu et al., 2021). Nevertheless, an increasing body of scholarly investigation suggests that reference genes do not consistently demonstrate stable expression across all circumstances, and the arbitrary selection of reference genes may yield unreliable experimental data (Liu et al., 2022). In studies involving multiple species such as *Brassica rapa* L. (Ma et al., 2020); *Momordica charantia* (Wang et al., 2019); *Stellera chamaejasme* (Liu et al., 2018); celery (Feng et al., 2019); *Fragaria chiloensis* (Gaete-Eastman et al., 2022); *Bromus sterilis* (Sen et al., 2021), and barley (Cai et al., 2018; Walling et al., 2018), the results of identifying reference genes indicate that for the same species under different conditions or different species under the same conditions, the identification of reference genes may different (Škiljaica et al., 2022). Hence, the selection of the most suitable housekeeping gene as the internal reference gene for RT-qPCR analysis in various experimental treatments holds significant importance in obtaining accurate gene expression results.

In this study, the selection of seven candidate genes was informed by literature reports. Notably, *Alpha tubulin -1 (TUB1)* and *Elongation factor 1-alpha (EF1α)* demonstrated acceptable stability when subjected to NaCl and hormone treatment in quinoa, as observed by Zhu (Zhu et al., 2021). Additionally, the investigation of saponins in quinoa leaves involved the utilization of *Vacuolar fusion protein MON1 (MON1)* as an internal reference gene for qPCR analysis, as reported by Fiallos-Jurado et al. (2016). In the study conducted by (Wang et al., 2022b) it was found that *Actin-1 (ACT)* emerged as the most stable reference gene when subjecting *Neoscytalidium dimidiatum* under different temperatures.

Similarly, Wang et al. (2022a) observed that *Glyceraldehyde-3-phosphate dehydrogenase A (GAPDH)* demonstrated the highest stability in the in vitro proliferation of satellite cells derived from bovine skeletal muscle. Furthermore, the selection of internal reference genes, such as *Beta tubulin -6 (TUB6)* and *Eukaryotic translation initiation factor 3 (EIF3)*, is influenced by the variation in species and conditions (Shi et al., 2012; Taki and Zhang, 2013; Bevitori et al., 2014). To assess the stability of these internal reference genes, GeNorm (Vandesompele et al., 2002), NormFinder (Wang et al., 2022a), BestKeeper (Pfaffl et al., 2004; Wang et al., 2022a), RefFinder (Taki and Zhang, 2013), Cycle threshold (Ct) value, and Delta Ct were employed (Ruduś and Kępczyński, 2018).

In order to enhance the precision and replicability of the experiment, the present study adhered to the guidelines outlined in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin et al., 2009). Presently, the extent of research concerning reference genes in quinoa is considerably limited, primarily centered around the assessment of candidate genes in various tissues or plants subjected to hormone treatments. Nevertheless, this investigation distinctively emphasizes the identification of internal reference genes in quinoa seeds throughout the process of natural germination and exogenous hormone treatments. Consequently, this research is anticipated to make a valuable contribution to the exploration of quinoa germination dormancy, PHS, and forthcoming systematic molecular biology studies in quinoa.

MATERIAL AND METHODS

Materials and exogenous hormone treatment

The experimental material utilized in this study was the H1 cultivar of quinoa, which was obtained from the Key Laboratory of Coarse Cereal Processing, Ministry of Agriculture and Rural Affairs P.R. China. The quinoa samples were stored at a temperature of 4 °C. In order to examine the impact of exogenous hormone treatment on seed germination, precise measurements of 0.0661 g and 0.0866 g of ABA and GA, respectively, were obtained from storage at -20 °C. Afterwards, the samples were placed into individual containers containing a small amount of anhydrous ethanol and mixed until complete dissolution of ABA/GA occurred. The resulting solutions were then diluted with ultrapure water to a final volume of 250 mL, resulting in a stock solution of 1 mM ABA and GA. Subsequently, ABA and GA solutions were prepared by serially diluting the stock solution to concentrations of 200 µM, 100 µM, 50 µM, and 10 µM. Plump quinoa seeds, chosen based on their visual characteristics, were immersed in 70 % ethanol for a duration of 20 s. The seeds were then rinsed three times with water and any excess surface moisture was absorbed using filter paper. A set of 100 seeds were subsequently positioned on a circular glass Petri dish, which possessed a radius of 4.5 cm, and was equipped with germination filter paper. Each Petri dish was filled with 6mL of a hormone solution that had been prepared with different concentrations. In the control group, an equivalent volume of H_2O was introduced. The germination filter paper and seeds were diligently maintained in a moist state. Each treatment was replicated three times biologically. The petri dishes were subsequently placed in a constant temperature chamber (Jiangnan, Zhejiang, China) with a light/dark cycle of 16 h light and 8 h darkness, maintaining a temperature of 25 °C during the day and 22 °C during the night, a relative humidity of 50%, and a light intensity of 40% (Tang et al., 2022). After 48 h, the seeds were washed three times with water. Fresh filter paper was inserted into the petri dishes, and 6 mL of ultrapure water was added to each plate. The plates were then incubated for an additional 24 h under the same conditions.

The germination standard was operationally defined as the point at which the quinoa sprout attains 50 % of the seed width (Tang et al., 2022). The initial germination stage spans from 0 to 48 h, during which data is collected at 4-hour intervals. Subsequently, in the second stage, spanning from 48 to 72 h, data is collected at 12-hour intervals. At each data collection point, the germinated seeds are carefully extracted from the culture dish. The calculation formulas for germination rate (GR) (Metwally et al., 2022), germination potential (GP) (Wang et al., 2023), germination index (GI) (Aloui et al., 2014; Chen et al., 2018), mean germination time (MGT) (Ullah et al., 2022; Mohanty et al., 2023), and peak germination (PG) (Arnolds et al., 2015) are provided below.

$$GR(\%) = (G_{n1}/N) \times 100$$

$$GP(\%) = (G_{n2}/N) \times 100$$

$$GI = \sum (G_t/T_t))$$

$$MGT = (\sum (Gt * Tt))/G_{n1}$$

$$PG = max(Gt/Tt)$$

In the context of formulas, G_{n1} represents the count of seeds that have germinated after a 72-hour period, while G_{n2} represents the count of seeds that have germinated within a 12 to 16-hour timeframe. N denotes the total number of seeds, G_t signifies the count of seeds that have germinated between the time intervals t and t-4 hour, and Tt represents the duration in t hours. The maximum quotient of G_t/T_t is denoted as PG. The determination of the optimal treatment concentration was made by considering the outcomes of the conducted experiments.

A total of 0.5 g of quinoa seeds were accurately measured and subsequently treated with 6 mL of ABA/GA/H_2O in separate petri dishes. The concentrations of ABA/GA utilized in the treatment were determined based on the concentrations established in the preceding experiment. The experimental conditions were upheld as previously outlined. Each treatment was replicated three times using distinct biological samples. Sampling was conducted at 4 h, 12 h, 20 h, 28 h, 36 h, and 44 h. The seeds' surface moisture was absorbed using filter paper, followed by their wrapping in aluminum foil and subsequent freezing in liquid nitrogen. The frozen seeds were then stored at a temperature of -80 °C to facilitate the subsequent extraction of RNA.

RNA extraction, first strand cDNA synthesis and primer design

The material was removed from the freezer at a temperature of -80 °C, pulverized in a mortar with the addition of liquid nitrogen, and a quantity of 50 mg of the resulting powder was utilized for the extraction of RNA. The extraction of RNA from all samples was performed using a plant tissue extraction kit (Tiangen, Beijing, China). Following this, the integrity and concentration of the RNA were evaluated using the Scan Drop100 high-throughput protein concentration meter (Analytik jena AG, Jena, Germany) by measuring the absorbance at wavelengths of 260 nm and 280 nm. The A260 / A280 ratio, which is typically between 1.8 and 2.2, is commonly used as an indicator of RNA purity and concentration. Furthermore, a 50 M TAE buffer (pH=8.0-8.6, Sangon Biotech, Shanghai, China) was diluted to 1 M TAE for the purpose of gel electrophoresis of nucleic acids. The gel electrophoresis was performed using a 1.00 % agarose gel in 1 M TAE buffer, and GoldviewTM dye (ZOMANBIO, Beijing, China) was utilized as the staining agent. The electrophoretic results were observed using a gel imaging system (BIO RAD, California, U.S.A) to validate the RNA quality based on established experimental criteria. Following the confirmation of RNA quality, the first-strand cDNA synthesis was conducted using the FastKing RT Kit (with gDNase, Tiangen, Beijing, China). The gDNA removal system was prepared by combining 2 µL of 5× gDNA Buffer, 1 µg RNA, and RNase-Free ddH_2O to achieve a final volume of 10 µL. The resulting mixture was thoroughly mixed and incubated at 42 °C for 3 m, followed by immediate placement on ice. For the reverse transcription system, a total volume of 10 µL was prepared by combining 2 µL of 10×King RT Buffer, 1 µL of FastKing RT Enzyme Mix, and 2 µL of FQ-RT Primer Mix, with the remaining volume supplemented with RNase-Free ddH_2O. The resulting mixture was combined with the gDNA removal system, and the subsequent reaction was carried out at a temperature of 42 °C for a duration of 15 minutes. This was followed by a denaturation step at 95 °C for 3 m, resulting in the synthesis of the first chain cDNA.

Following a comprehensive literature review, a total of seven potential reference genes, namely *TUB1, TUB6, EIF3, EFIα, GAPDH, MON1,* and *ACT,* were chosen for the purpose of validation. The sequences of the target genes that underwent successful validation were sourced from the Gene Database of the National Center for Biotechnology

Information (NCBI) available at https://www.ncbi.nlm.nih.gov. The protein sequences were employed as templates for conducting BLAST searches to ascertain homologous sequences in quinoa. The homologous sequences that were obtained through downloading were chosen based on alignment metrics, including total score, query cover, E-value, and percent identity. Subsequently, primers were devised to span exonic regions, with lengths varying from 80 to 200 bp and GC falling within the range of content between 40% to 60%. The design of these primers was accomplished through the utilization of NCBI-primer-Blast and Primer Premier 5.0.

RT-qPCR assay

The samples were acquired from three biological replicates. The double-stranded cDNA synthesis process was monitored using the M5 Hiper SYBR Premix EsTaq with Tli RNaseH (Mei5 Bio, Beijing, China). The RT-qPCR was performed on white 96-well plates using the qTOWER3 G instrument (Analytik jena AG, Jena, Germany). Each reaction was prepared using a 20 µL system, consisting of 1 µL of diluted cDNA (with an approximate concentration of 80 ng.µL^{-1}), 0.4 µL of each forward and reverse primer (100 µM), and 10 µL of 2*M5 Hiper SYBR Premix EsTaq (containing Tli RNaseH). The total volume was adjusted to 20 µL by adding additional ddH_2O. The RT-qPCR program comprised a pre-denaturation step at 95 °C for 30 s, followed by a reaction stage at 95 °C for 5 s, and 60 °C for 20 s, repeated for a total of 40 cycles. Melting curves were generated by gradually increasing the temperature from 60 °C to 95 °C at a rate of 5 °C per second to confirm the specificity of the primers used for amplification. Additionally, no template controls (NTC) were included in the experimental process, and no amplification was observed in the NTC, indicating the absence of primer dimer formation.

Amplification efficiency and stability analysis

A fivefold dilution process was applied to the cDNA samples to produce templates with diverse concentrations. The amplification efficiency was subsequently determined using the formula E (%) = (10^ (-1 / slope) - 1) * 100, as described by (Wang et al., 2022a) and (Migocka and Papierniak, 2011), with a qualification of 90-110 % amplification efficiency. Additionally, the primer amplification efficiency was further validated in this study using LinRegPCR, as outlined by (Borges et al., 2012).

The stability of candidate genes was calculated by methods, including Ct values, geNorm, NormFinder, BestKeeper, RefFinder, and Delta Ct. The analysis of Ct values involved the plotting of all values to determine the maximum, minimum, percentiles, and medians, thereby providing an initial assessment of the dispersion of reference gene expression. It is important to note that the NormFinder, BestKeeper, RefFinder, and Delta Ct utilized in this study are R packages for Windows, while geNorm is a software. Each of these approaches employs unique calculation methodologies.

Before performing geNorm analysis, the Ct values were normalized using the $2^{-\Delta Ct}$ method, where $\Delta Ct = (Ct - Ct_{min})$ (Wang et al., 2019). GeNorm (Vandesompele et al., 2002) evaluates the stability of potential reference genes by comparing their expression levels to those of other candidates, utilizing the standard deviation of the logarithmic ratios of the disparities between them. The stability of candidate genes is assessed by the M value, where a smaller M value signifies greater stability. The impact of including an additional reference gene on the results is evaluated using the paired variation value Vn/(Vn+1) in geNorm, where a Vn/(Vn+1) value exceeding 0.15 suggests the requirement for more reference genes. Conversely, an Vn/(Vn+1) value below 0.15 suggests a negligible impact from the addition of another reference gene. NormFinder was employed to compute the S value for candidate genes, where a smaller S value indicates higher stability of the gene. The algorithms employed by BestKeeper rely on Cross Pinot (CP), a method that identifies optimal reference genes by conducting pairwise correlation analysis and calculating the geometric mean of sample pairs (Pfaffl et al., 2004). BestKeeper further computes various statistical measures, including the Geometric Mean (GM), Arithmetic Mean (AM), Minimal value (Min), Maximal value (Max), Standard Deviation (SD), and Coefficient of Variance (CV), based on the sample data. The stability of candidate genes increases as the coefficient of variation (CV) and standard deviation (SD) decrease. The Delta Ct method, as proposed by Hu et al. (2014), identifies reference

genes with stable expression by comparing the relative expression levels of genes in the samples. However, it should be noted that there is not uniform consistency among all treatment groups in terms of the results obtained from different tools. To address this issue, RefFinder employs a weighting system for the calculation methods utilized by various tools mentioned. This enables the provision of an overall ranking of candidate reference gene stability.

Based on the aforementioned analysis findings, the two most stable candidates and the least stable candidate gene were chosen as reference genes. *ABA8ox1 (AUR62030408-RA)* and *GA2ox1 (AUR62002752-RA)*, which are metabolic genes associated with ABA and GA, were designated as target genes. The $2^{-\Delta\Delta Ct}$ method was employed to quantify their expression levels. The RT-qPCR system and program used were consistent with those described in section 'RT-qPCR assay'. To verify the dependability of the reference gene selection outcomes, the expression levels were assessed separately under different treatments.

Statistical analysis

Graphs were created using GraphPad Prism 8 and Origin 2023. Mean, standard deviation, and one-way ANOVA were analyzed using IBM SPSS Statistics 26. Amplification efficiency was calculated using Analytik Jena software, in conjunction with Excel 2016 and LinRegPCR.

RESULTS AND DISCUSSION

The germination rates under various treatments were observed and recorded in Figure 1 within a 72-hour time frame. At the 48-hour mark, the CK group exhibited an average germination rate of 98% ± 1%. In the ABA group, the germination rates at concentrations of 10 µM, 50 µM, 100 µM, and 200 µM were 98 % ± 1%, 63% ± 4%, 18% ± 3%, and 10% ± 2%, respectively. The germination rates of the GA group at concentrations of 10 µM, 50 µM, 100 µM, and 200 µM were determined to be 95% ± 1%, 98% ± 1%, 98% ± 2%, and 96% ± 0%, respectively. Comparatively, the germination rates of the ABA group at concentrations of 50 µM, 100 µM, and 200 µM were observed to be lower than those of both the CK and GA groups after 48 h. However, no statistically significant difference was found between the germination rates of the GA and CK groups. Following the completion of the initial 48-hour stage, the seeds were

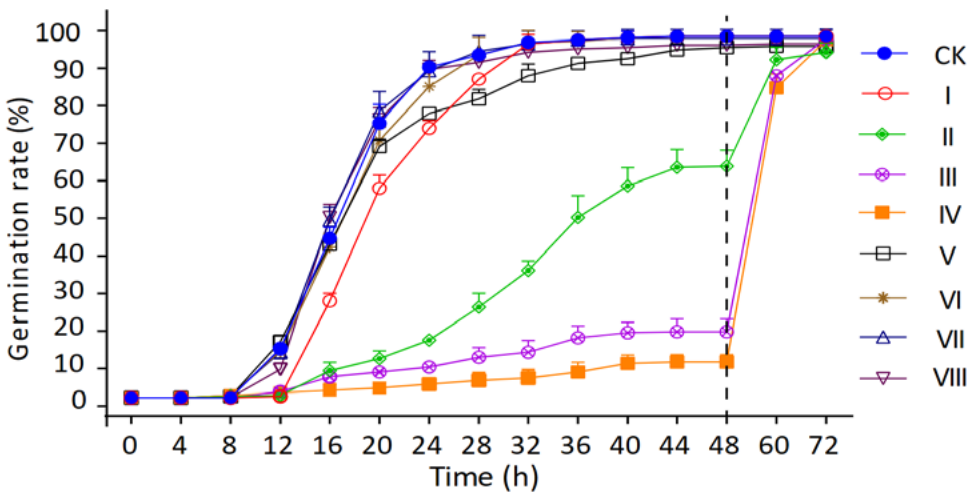

Figure 1. The statistical analysis conducted in this study examined the germination rates of seeds subjected to treatments involving H_2O, ABA, and GA within a 72-hour period. The control group, denoted as CK, was treated with H_2O. ABA treatments were categorized as I to IV, representing concentrations of 10 µM, 50 µM, 100 µM, and 200 µM, respectively. Similarly, GA treatments were categorized as V to VIII, representing concentrations of 10 µM, 50 µM, 100 µM, and 200 µM, respectively.

subsequently transferred into petri dishes containing water. During the subsequent 24-hour period, the groups treated with ABA at concentrations of 50, 100, and 200 µM, which had previously displayed lower germination rates in the first stage, demonstrated a rapid attainment of complete germination. Table 1 presents the average values, standard deviations, and results of a one-way ANOVA for the seed vigor-related indicators PG, GP, GI, and MGT in the CK, ABA, and GA groups. In the ABA group, significant differences were observed in all indicators at 50 µM, 100 µM, and 200 µM compared to the CK group. Conversely, in the GA group, no significant differences were found at 10 µM and 50 µM compared to the CK group. However, significant differences were observed in PG and GP at 100 µM and 200 µM, while MGT and GI did not show significant differences.

After treatment with four concentrations of hormone (10 µM, 50 µM, 100 µM, and 200 µM) for 24 h, there was no significant effect observed on the germination of quinoa seeds when treated with 10 µM ABA (Figure 2A).

Table 1. Statistics of seed germination vitality indicators.

	PG	GP	GI	MGT
CK	1.875±0.165 c	0.300±0.026 c	5.458±0.050 ab	19.317±0.524 ef
I	1.612±0.099 c	0.257±0.015 c	4.704±0.109 c	22.211±0.349 d
II	0.483±0.060 e	0.067±0.012 d	2.742±0.210 d	41.019±1.509 c
III	1.161±0.042 d	0.040±0.010 de	2.021±0.099 e	53.947±1.610 b
IV	1.244±0.051 d	0.007±0.006 e	1.817±0.182 e	58.192±0.96 9a
V	1.646±0.036 c	0.263±0.006 c	5.185±0.077 b	20.855±0.895 de
VI	1.771±0.130 c	0.283±0.021 c	5.371±0.223 ab	19.843±0.765 ef
VII	2.250±0.225 b	0.360±0.036 b	5.536±0.188 a	18.859±0.525 f
VIII	2.583±0.253 a	0.413±0.040 a	5.337±0.093 ab	19.252±0.552 ef

Perform significance analysis based on the $P \leq 0.05$ level. PG: Peak germination, GP: germination potential, GI: germination index, MGT: Mean germination time; From I to IV represents ABA 10 µM, 50 µM, 100 µM, 200 µM; From V to VIII represents GA 10 µM, 50 µM, 100 µM, 200 µM.

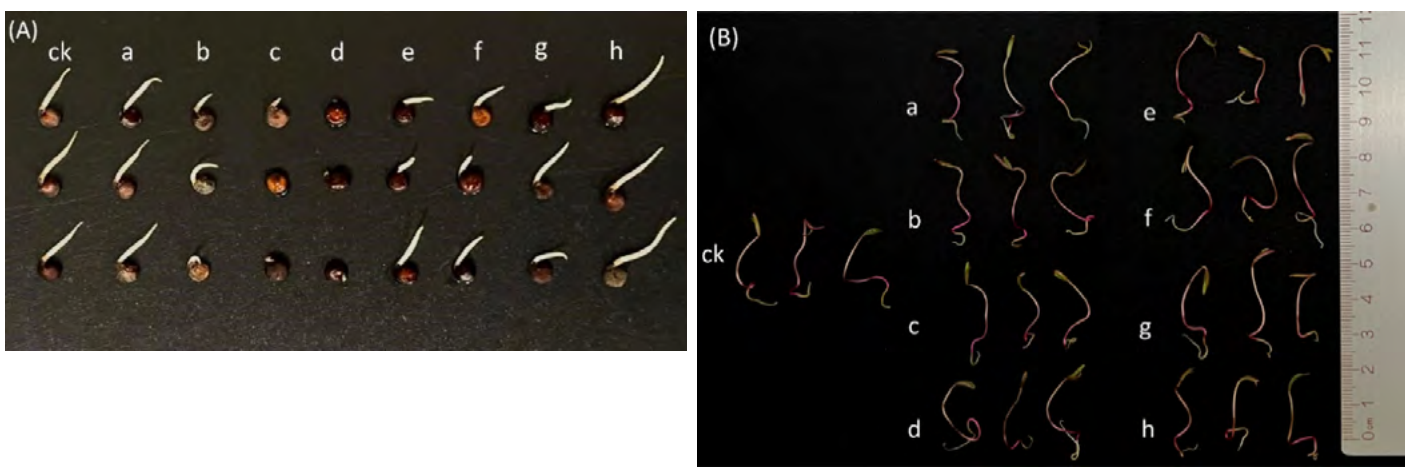

Figure 2. Quinoa seeds were subjected to various concentrations of ABA and GA. The germination state at 48 hours was denoted by (A), while the group treated with H_2O was represented by "ck". Columns a, b, c, and d corresponded to treatments with concentrations of 10 µM, 50 µM, 100 µM, and 200 µM ABA, respectively. Columns e, f, g, and h referred to GA treatments with concentrations of 10 µM, 50 µM, 100 µM, and 200 µM, respectively. (B) depicts images of seedlings subjected to various treatments. The seedling states on the 5th day of cultivation with water and varying concentrations of GA are denoted by ck, e, f, g, and h, while the states following the transfer of ABA to water culture for 3 days are represented by a, b, c, and d.

When seeds were subjected to 50 µM ABA treatment, shoot elongation exhibited a decrease compared to the CK group. Furthermore, at concentrations of 100 µM and 200 µM, germination was suppressed. Conversely, from the perspective of shoot length, no significant impact on seed germination was observed from treatment with 10 µM, 50 µM, and 100 µM GA. Notably, at a GA concentration of 200 µM, seed shoot elongation exhibited a discernible increase. The sustained germination process indicates the potential for successful seedling development (Figure 2B). In summation, for subsequent treatments, GA was administered at a concentration of 200 µM, while ABA was applied at a concentration of 100 µM.

Following the implementation of 1% agarose gel electrophoresis on each of the experimental RNA samples, the outcomes were visually presented in Figure 3. The assessment of RNA and cDNA purity and concentration was performed using Scan Drop. The study revealed that the A260/A280 ratio of the RNA samples varied between 1.82 and 2.21, while the purity of the cDNA ranged from 1.78 to 1.85 (Table 2). Detailed descriptions of the candidate gene primers were provided, specifying a maximum primer length of 197 bp and a minimum primer length of 121bp (Table 3). The melting curves of all candidate genes exhibited a unimodal peak shape, as illustrated in Figure 4. The amplification efficiency of the primers, as presented in Table 3, fell within the acceptable range of 90% to 110%, except for the *EF1α* primer, which was excluded due to its deviation from the defined range. The efficiencies of the remaining primers ranged from 94.17% to 110.17%, and their amplification curves demonstrated a strong linear relationship, as evidenced by the R^2 values ranging from 0.9853 to 0.9996. The amplification efficiency during revalidation using LinRegPCR varied between 102.8% and 110.3% as indicated in Table 3.

The Ct value is used to reflect the transcription level of a gene in mRNA, with a lower Ct value indicating a higher transcription level (Tang et al., 2017). In Figure 5, the maximum Ct value for *GAPDH* among all samples was 30.47, while the minimum Ct value for *TUB1* was 17.49. The Interquartile range (IQR), analysis revealed that, as for *GAPDH,* the three treatments exhibited a relatively large dispersion, ranging from 19.11 to 30.47. In contrast, *MON1* and *EIF3* showed a more concentrated distribution, with Ct values ranging from 21.83 to 27.02 and 19.97 to 25.6, respectively.

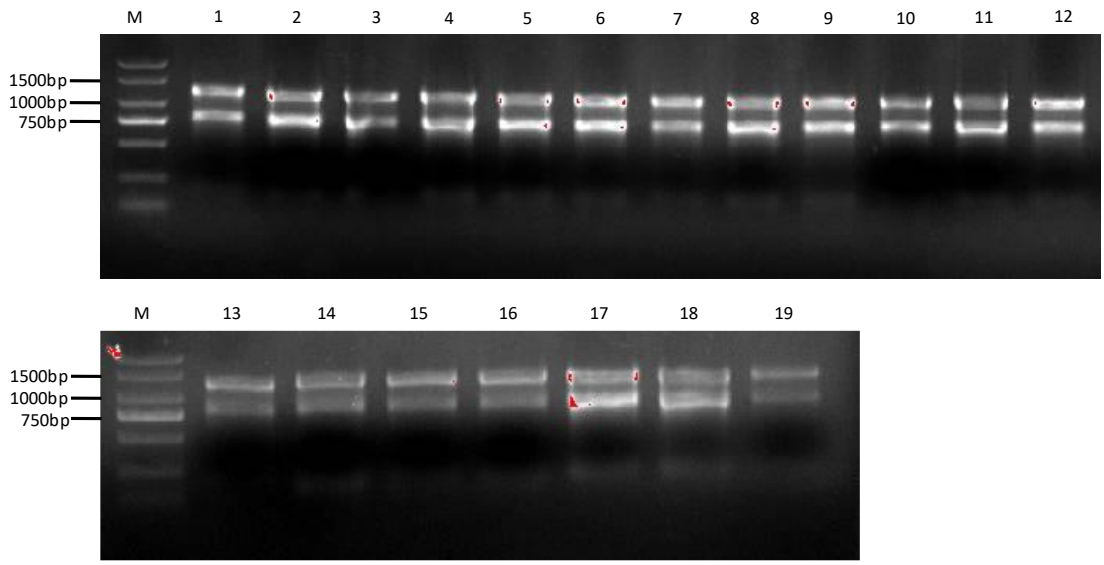

Figure 3. RNA agarose gel electrophoresis was performed on samples subjected to various treatments. Lanes 1-6 contained RNA extracted from samples treated with ABA for 4 h, 12 h, 20 h, 28 h, 36 h and 44 h, respectively.; Lanes 7-12 represented RNA obtained from samples treated with GA for the same time intervals. Lanes 13-18 contained RNA collected from samples treated with H_2O for 4 h, 12 h, 20 h, 28 h, 36 h, and 44 h, respectively. Lane 19 contained RNA from samples collected at 0 h. A 2000 bp marker was used for reference.

Table 2. Purity and concentration of RNA /cDNA.

Treatment	Time	RNA		cDNA	
		A260/A280	Concentration ng.µL^{-1}	A260/A280	Concentration ng.µL^{-1}
	0h	1.93	165.69	1.81	923.67
GA	4 h	1.82	66.55	1.8	964.32
	12 h	1.97	216.35	1.83	1005.96
	20 h	1.95	325.37	1.83	1029.58
	28 h	2.21	346.33	1.81	882.77
	36 h	2.17	723.87	1.85	1043.86
	44 h	2.13	537.51	1.79	852.25
ABA	4 h	2.13	76.43	1.82	960.73
	12 h	2.01	210.92	1.81	983.06
	20 h	1.96	364.58	1.8	968.48
	28 h	2.16	612.26	1.84	975.35
	36 h	2.17	539.78	1.8	890.02
	44 h	2.15	344.11	1.78	887.63
H_2O	4 h	1.88	52.63	1.81	964.96
	12 h	2.03	208.75	1.78	936.43
	20 h	2.02	338.04	1.84	1037.34
	28 h	2.15	442.07	1.85	998.2
	36 h	2.15	537.98	1.84	974.3
	44 h	2.16	538.86	1.83	1004.76

Table 3. Candidate reference genes characteristics and primer sequences information.

Gene name	Description	Gene ID	Amplicon length (bp)	Primer sequences	Slope	Intercept	R^2	Efficiency	
				forward /reverse(5´to3´)				Analytik Jena	LinRegPCR
ACT	*Actin-1*	110715281	173	AGCTTCTTGACGAAATGGGTG ACAACTCCTCACCTTCTCATGG	-3.38	28.31	0.9996	97.63%	110.30%
TUB6	*Beta tubulin -6*	110711758	157	GGAAAAATGAGAGAAATCCTTCACA AGTAGACATTGACGCGCTCC	-3.35	29.79	0.9853	98.84%	105.60%
TUB1	*Alpha tubulin -1*	110686159	144	GTGCTCCTGTATGGTGCCAA	-3.1	27.09	0.9986	110.17%	102.80%
EIF3	*Eukaryotic translation initiation factor 3*	110711181	121	AGCGGACCAACAAACAGAAAAG AGATCGATGTTGAGGCGAGC	-3.45	30.34	0.9914	94.92%	103.75%
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase A*	110711214	197	GGAAAAATGAGAGAAATCCTTCACA TCTTCCACAAGGCCAAGGAA	-3.19	31.33	0.9961	105.82%	108.62%
MON1	*Vacuolar fusion protein MON1*	110720838	147	TGCGAAGCCAGCGTAGTTTA GCTGGCTGCAGCTCAATATAC	-3.47	31.27	0.9906	94.17%	103.85%

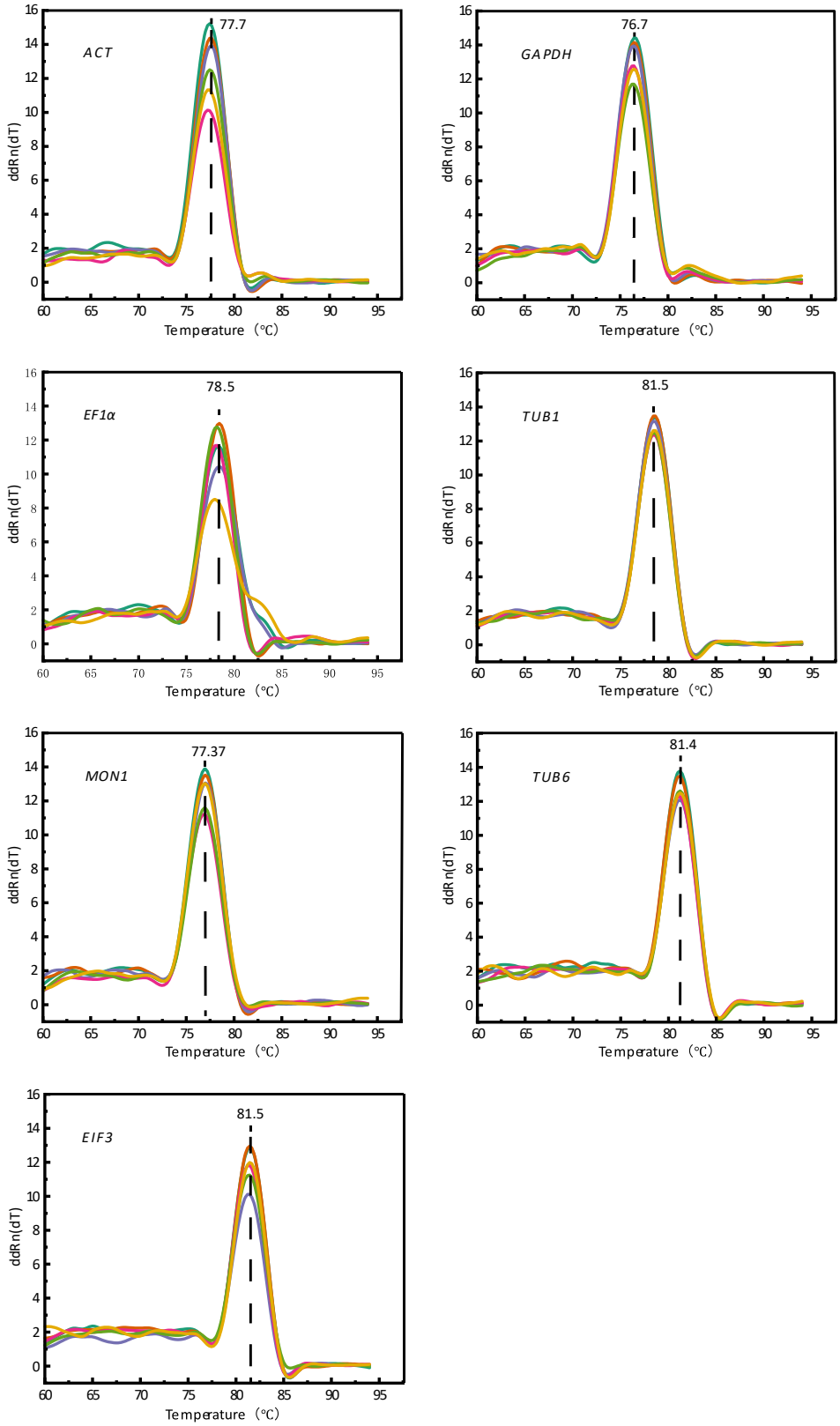

Figure 4. The melting curves of candidate internal reference genes. The temperature indicated by the dashed line is the gene Tm value.

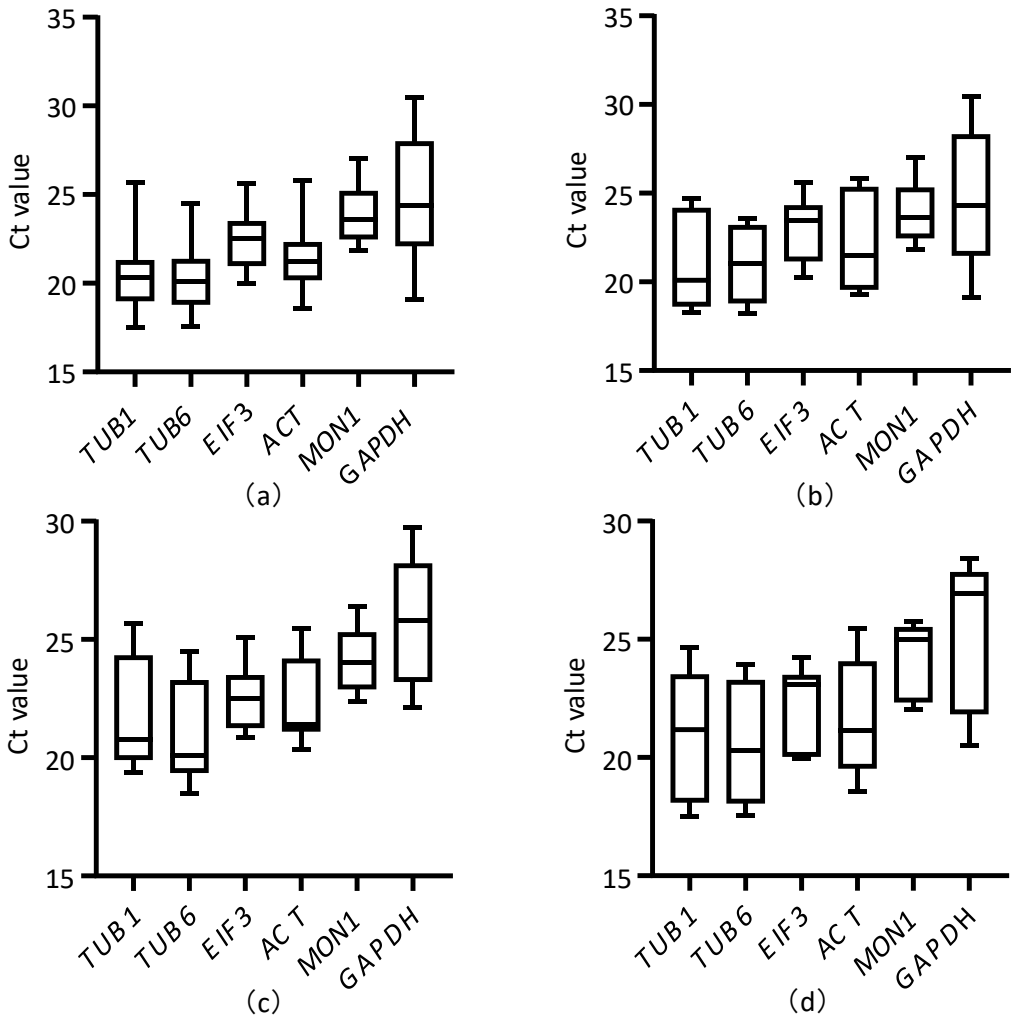

Figure 5. The expression parameters of candidate genes are depicted using a box plot, where the box represents the interquartile range. The lower and upper boundaries of the box represent the 25th and 75th percentiles, respectively, while the line inside the box indicates the median. Specifically, (a) represents the Ct values of all treatments, (b) corresponds to the H_2O treatment group, (c) represents the ABA treatment group, and (d) refers to the GA treatment group.

According to the boxplot analysis, the H2O treatment group, the medians of *TUB1, ACT, MON1*, and *GAPDH* are positioned towards the lower end, while the median of *TUB6* falls in the middle. The average Ct value of *TUB1* in this group was the lowest, measuring at 20.92. In contrast, the ABA and GA treatment groups displayed the smallest IQR and the lowest dispersion for *MON1* and *EIF3. TUB6* demonstrated the lowest average Ct value in both hormone treatment groups, with a Ct value of 20.88 in the ABA treatment group and 20.51 in the GA treatment group.

The stability of six candidate genes was assessed using geNorm analysis (Table 4). In the H_2O group, *ACT* and *TUB6* exhibited the highest stability (M = 0.075), while *GAPDH* demonstrated the lowest stability (M = 0.225). Conversely, in the ABA treatment group, *TUB6* and *ACT* were identified as the most stable genes (M = 0.068), whereas in the GA treatment group, *ACT* and *GAPDH* displayed the highest stability (M = 0.072), with *TUB6* being the least stable. When considering all treatments as a single group, geNorm analysis revealed that *TUB1* and *ACT* exhibited high stability, as indicated by an M value of 0.097, whereas *GAPDH* demonstrated the lowest stability with an M value of 0.213. The determination of the appropriate number of reference genes was based on the paired variation values (Figure 6), which were 0.0295 (H_2O), 0.0276 (ABA), 0.0494 (GA), and 0.0446 (all groups) for V2/3.

Table 4. Analysis results and stability ranking by geNorm, NormFinder, Delta Ct, RefFinder.

Treatment	Rank	geNorm		NormFinder		Delta Ct		RefFinder	
		Gene	mean M	Gene	S value	Gene	Stability	Gene	Stability
H_2O	1	*TUB6*	0.075	*TUB1*	0.21	*ACT*	1.18	*ACT*	1.68
	2	*ACT*	0.075	*ACT*	0.43	*TUB1*	1.19	*TUB1*	1.78
	3	*EIF3*	0.088	*MON1*	0.51	*TUB6*	1.2	*MON1*	2.63
	4	*MON1*	0.097	*TUB6*	0.68	*MON1*	1.21	*TUB6*	3.22
	5	*TUB1*	0.103	*EIF3*	1.19	*EIF3*	1.49	*EIF3*	3.98
	6	*GAPDH*	0.225	*GAPDH*	2.51	*GAPDH*	2.58	*GAPDH*	6
ABA	1	*TUB6*	0.068	*TUB6*	0.28	*TUB6*	0.83	*MON1*	1.68
	2	*ACT*	0.068	*MON1*	0.56	*MON1*	0.91	*EIF3*	1.73
	3	*TUB1*	0.082	*EIF3*	0.67	*EIF3*	0.96	*TUB6*	1.86
	4	*MON1*	0.091	*TUB1*	0.67	*TUB1*	0.98	*ACT*	4.16
	5	*EIF3*	0.098	*ACT*	0.71	*ACT*	1.01	*TUB1*	4.47
	6	*GAPDH*	0.154	*GAPDH*	1.34	*GAPDH*	1.47	*GAPDH*	6
GA	1	*ACT*	0.072	*EIF3*	0.65	*EIF3*	1.15	*EIF3*	1.19
	2	*GAPDH*	0.072	*TUB1*	0.66	*TUB1*	1.19	*MON1*	1.73
	3	*TUB1*	0.126	*MON1*	0.83	*MON1*	1.23	*TUB1*	2.78
	4	*EIF3*	0.157	*ACT*	0.86	*ACT*	1.27	*ACT*	4
	5	*MON1*	0.189	*TUB6*	1	*TUB6*	1.35	*TUB6*	4.4
	6	*TUB6*	0.227	*GAPDH*	1.57	*GAPDH*	1.76	*GAPDH*	6
All treatments	1	*TUB1*	0.097	*TUB1*	0.51	*MON1*	1.11	*MON1*	1.19
	2	*ACT*	0.097	*MON1*	0.58	*ACT*	1.13	*EIF3*	2.51
	3	*EIF3*	0.127	*ACT*	0.62	*TUB1*	1.14	*ACT*	2.91
	4	*MON1*	0.149	*EIF3*	0.75	*TUB6*	1.17	*TUB1*	2.94
	5	*TUB6*	0.164	*TUB6*	0.76	*EIF3*	1.17	*TUB6*	3.94
	6	*GAPDH*	0.213	*GAPDH*	1.98	*GAPDH*	2.08	*GAPDH*	6

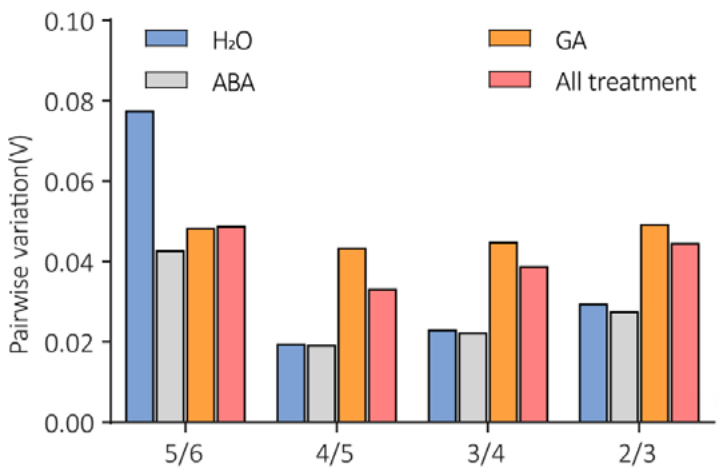

Figure 6. The geNorm method was employed to determine the pairwise variation (V) of candidate reference genes.

These findings demonstrate that employing the top-ranked two candidate genes as internal reference genes resulted in unaffected experimental normalization when a third or more internal reference genes were added. Consequently, it is recommended that the utilization of two internal reference genes is adequate, obviating the necessity of introducing additional internal reference genes.

NormFinder was employed to calculate the S value for six candidate genes (Table 4). The stability ranking assigned by NormFinder for various liquid treatments of quinoa seeds exhibited dissimilarity. Among the quinoa grain samples treated with H_2O, the stability ranking of the candidate genes, in descending order, was as follows: *TUB1*, *ACT*, *MON1*, *TUB6*, *EIF3*, and *GAPDH*, with *TUB1* exhibiting the lowest S value of 0.21. In the ABA-treated group, the candidate genes exhibited a stability ranking of *TUB6*, *MON1*, *EIF3*, *TUB1*, *ACT*, and *GAPDH*, with S values ranging from 0.28 to 1.34. Notably, TUB6 displayed the lowest S value of 0.28. Conversely, in the GA-treated group, the stability ranking of the candidate genes was *EIF3*, *TUB1*, *MON1*, *ACT*, *TUB6*, and *GAPDH*, with *EIF3* exhibiting an S value of 0.65. In terms of the comprehensive analysis findings across the three treatment groups, the stability ranking is as follows: *TUB1, MON1, ACT, EIF3, TUB6, GAPDH*. The highest-ranked candidate gene, *TUB1*, exhibits an S value of 0.51. NormFinder consistently identified *GAPDH* as the least stable candidate gene across all groups. In three conditions, the gene *GAPDH* consistently exhibited the lowest level of stability.

The data from four sets were subjected to analysis using BestKeeper. The sample sizes for the H_2O, ABA, and GA groups were 21, while the fourth group, encompassing all treatments, had a sample size of 57. Table 5 presents the GM, AM, Min, Max, SD, and CV. The results of the study revealed that *MON1* and *EIF3* were identified as the most stable genes across all four categories, as indicated in Table 5. The CV values, accompanied by the SD values, for *MON1* and *EIF3* in the H_2O, ABA, GA treatments, and for all treatments collectively were as follows: 5.79±1.39, 6.38±1.46; 4.88±1.18, 5.2±1.17; 6.09±1.47, 7.04±1.56; 5.55±1.33, 6.45±1.45; respectively. Notably, based on the analysis conducted using BestKeeper, *MON1* emerged as the most stable candidate gene for both H_2O and GA treatments, as well as for all treatments combined. *EIF3* was determined to be the most stable gene within the ABA group. Conversely, *GAPDH* was found to be the least dependable gene across all groups. Research by Wang et al. (2022a) has shown that in BestKeeper analysis, SD values less than 1 indicate higher stability. However, in this study, all SD values were less than 1, and this observation may be due to the sample size.

Table 5. Stability of candidate gene results by BestKeeper.

Treatment	Rank	Gene name	geo Mean	AR Mean	Min	Max	Standard deviation (SD)	Coefficient of variation (CV)
H_2O	1	*MON1*	24.02	24.08	21.83	27.02	1.39	5.79
	2	*EIF3*	22.85	22.91	20.28	25.6	1.46	6.38
	3	*TUB6*	20.68	20.77	18.19	23.58	1.71	8.25
	4	*ACT*	21.93	22.05	19.29	25.81	2.03	9.22
	5	*TUB1*	20.79	20.92	18.25	24.67	2.06	9.86
	6	*GAPDH*	24.44	24.73	19.11	30.47	3.4	13.74
ABA	1	*EIF3*	22.53	22.57	20.85	25.08	1.17	5.2
	2	*MON1*	24.09	24.13	22.36	26.41	1.18	4.88
	3	*ACT*	22.22	22.28	20.34	25.47	1.45	6.51
	4	*TUB6*	20.78	20.88	18.47	24.5	1.73	8.28
	5	*TUB1*	21.56	21.67	19.39	25.66	1.91	8.82
	6	*GAPDH*	25.69	25.82	22.12	29.76	2.35	9.12

Continue...

Table 5. Continuation.

Treatment	Rank	Gene name	geo Mean	AR Mean	Min	Max	Standard deviation (SD)	Coefficient of variation (CV)
GA	1	*MON1*	24.04	24.09	22.02	25.74	1.47	6.09
	2	*EIF3*	22.06	22.12	19.97	24.22	1.56	7.04
	3	*TUB6*	20.4	20.51	17.56	23.93	1.81	8.81
	4	*ACT*	21.35	21.46	18.56	25.47	1.87	8.72
	5	*TUB1*	20.61	20.76	17.49	24.67	2.11	10.18
	6	*GAPDH*	24.81	25.01	20.51	28.41	2.97	11.87
All treatments	1	*MON1*	23.91	23.96	21.83	27.02	1.33	5.55
	2	*EIF3*	22.37	22.43	19.97	25.6	1.45	6.45
	3	*ACT*	21.48	21.57	18.56	25.81	1.52	7.04
	4	*TUB6*	20.35	20.44	17.56	24.5	1.61	7.89
	5	*TUB1*	20.65	20.77	17.49	25.66	1.76	8.49
	6	*GAPDH*	24.67	24.88	19.11	30.47	2.94	11.82

The Delta Ct calculation results were presented in Table 4. Among the H_2O-treated samples, *ACT* and *TUB1* emerged as the most stable candidate reference genes, exhibiting stability values of 1.18 and 1.19, respectively. In the case of ABA treatment, *TUB6* and *MON1* were relatively stable candidate genes, displaying corresponding stability values of 0.83 and 0.91. Similarly, in the GA treatment, *EIF3* and *TUB1* were identified as the most stable candidate genes, with stability values of 1.15 and 1.19, respectively. When the three data treatments were consolidated into a single group, the Delta Ct calculation outcomes revealed that *MON1* and *ACT* exhibited the highest levels of stability, with values of 1.11 and 1.13, respectively. In all groups, the Delta Ct method consistently indicated that *GAPDH* was the least stable reference gene candidate.

RefFinder utilizes geNorm, NormFinder, BestKeeper, and Delta Ct calculations to assign distinct weights and re-evaluate the stability, resulting in a revised ranking. The stability rankings of potential reference genes in the H_2O treatment group were determined to be *ACT* > *TUB1* > *MON1* > *TUB6* > *EIF3* > *GAPDH*. Similarly, in the ABA-treated group, the stability rankings were found to be *MON1* > *EIF3* > *TUB6* > *ACT* > *TUB1* > *GAPDH*, while in the GA-treated group, the stability rankings were determined to be *EIF3* > *MON1* > *TUB1* > *ACT* > *TUB6* > *GAPDH*, as presented in Table 4. RefFinder analysis yielded a stability ranking of *MON1* > *EIF3* > *ACT* > *TUB1* > *TUB6* > *GAPDH* when the three treatments were examined collectively. The findings of Zhu (Zhu et al., 2021) demonstrated that *TUB1* and *EF1α* were identified as the most acceptable for Long Li N° 1 quinoa under salt stress and ABA treatment. Conversely, *GAPDH* exhibited the highest level of instability when subjected to salt stress, 200 µM ABA, and other treatments, using various tissues as RNA templates. However, our findings indicate that the *MON1* and *EIF3* genes exhibited the highest stability in the H1 variety when subjected to hormone treatments. Similarly, the *GAPDH* gene displayed low stability in the Long Li N° 1 quinoa variety. These variations in gene stability could be attributed to the inherent differences in cultivars and treated tissues.In all conditions, the GAPDH consistently exhibited the lowest level of stability, with the exception of the geNorm analysis of samples treated with GA, where it ranked second in terms of stability. These results align with previous studies(Ruduś and Kępczyński 2018; Sudhakar-Reddy et al. 2018), wherein diverse stability analysis methods yielded disparate outcomes.

Based on the findings of the study, the choice of candidate reference genes for quinoa seeds is influenced by the specific treatments applied. Specifically, under H_2O treatment, the genes *ACT* and *TUB1* were identified as the most stable. Conversely, in the 100 µM ABA and 200 µM GA treatments, the candidate genes *EIF3* and *MON1* exhibited

similar levels of stability. Furthermore, when the data from all three treatments were integrated and analyzed, *EIF3* and *MON1* emerged as the top two genes in terms of stability. *GAPDH* emerged as the least stable candidate genes across all groups. In order to ensure the credibility of the results, reference genes *ACT* and *TUB1*, which exhibited high stability in the presence of H_2O treatment, were chosen alongside *GAPDH*, which demonstrated the lowest stability, to ascertain the relative expression levels of the target genes *ABA8ox1* (TGCAGACAAAGTTAAAAAGTATGGT/AAATTTAGCTGCATCCGGGC) and *GA2ox1* (GTTGGTGACTCTTTGCAGGTG/TGTCAGCCAAAACCCTGTGT). Furthermore, the expression levels of the target genes were determined using *EIF3* and *MON1,* which are characterized by high stability, and *GAPDH*, a gene with low stability, as reference genes under ABA treatment.

The validation outcome of the reference genes, as depicted in Figure 7, demonstrated that the relative expression levels of the target genes remained relatively consistent when *ACT*, *TUB1*, and *ACT+TUB1* were employed as reference genes under the H_2O group. However, significant fluctuations were observed when the least stable candidate gene, *GAPDH*, was utilized as a reference gene. In the H_2O-treated samples of *ABA8ox1*, the control group at 0 h was established. The utilization of *ACT*, *TUB1*, and *ACT+TUB1* as reference genes revealed a pattern of small-scale fluctuation in expression (Figure 7a). The gene expression levels ranged from 0.599 as the lowest value to 1.725 as the highest value across different time intervals. Conversely, when *GAPDH* was employed as a reference gene, the expression levels exhibited a wider distribution, ranging from 0.280 to 7.568. In Figure 7b, when employing the more stable genes as reference genes for *GA2ox1*, the expression levels exhibited a pattern of first decreasing, then fluctuating within a small range, value ranging from 0.142 to 0.480, and all were observed to be down-regulated. Conversely, when *GAPDH*

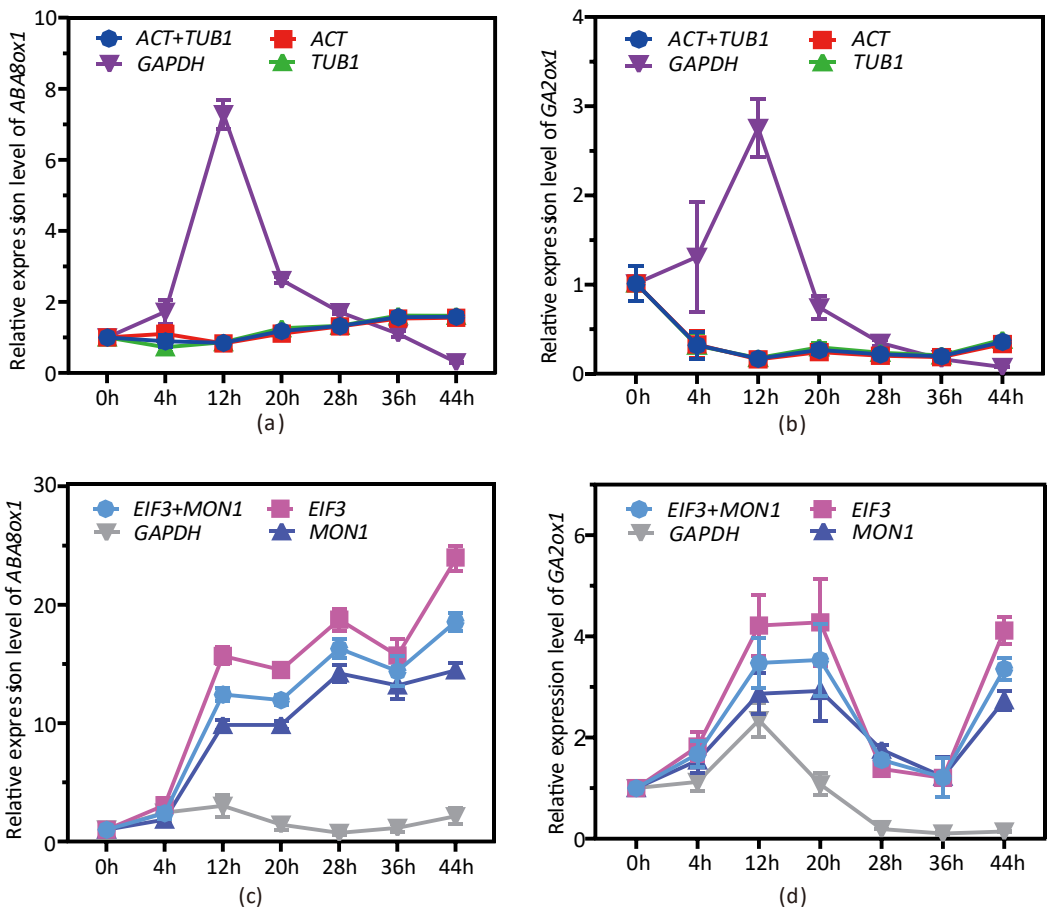

Figure 7. Validation results of stability of internal reference genes. (a), (b) represents the relative expression levels of *ABA8ox1* and *GA2ox1* in H_2O treatment, and (c), (d) refers to the relative expression levels of *ABA8ox1* and *GA2ox1* in ABA treatment, respectively.

was utilized as the reference gene, the gene expression levels ranged from 0.0735 to 3.053, displaying a contrasting trend compared to when *ACT*, *TUB1*, and *ACT+TUB1* were employed as reference genes, wherein an initial increase was followed by a subsequent decrease. Hence, the utilization of unstable genes as reference genes during the process of quinoa seed germination in the absence of external substances may lead to an overestimation of the actual expression levels.

Following the application of ABA treatment on quinoa seeds, the reference genes *EIF3*, *MON1*, and *EIF3+MON1* were employed, resulting in expression levels of *ABA8ox1* ranging from 1.491 to 24.533 when compared to the control group. Similarly, the expression levels of *GA2ox1* ranged from 0.891 to 5.169 (Figures 7c and d). When *GAPDH* was used as a reference gene, the expression level of *ABA8ox1* ranged from 0.522 to 3.971, while the minimum and maximum expression levels of *GA2ox1* were 0.0761 and 2.707, as depicted in Figure 7 c and d, respectively. When utilizing genes with high stability as reference genes, the *ABA8ox1* trend in the ABA treatment group exhibited a gradual increase from 0 to 44 h. Conversely, the utilization of the less stable *GAPDH* gene displayed a distinct trend. Similarly, when employing *EIF3*, *MON1*, and *EIF3+MON1* as reference genes, the expression trend of *GA2ox1* remained consistent from 0 to 44 h, while diverging from that of *GAPDH*. During the validation process of reference gene stability, it was observed that when either *MON1* or *EIF3* was used as the sole reference gene in the hormone-treated group, the expressing trend remained consistent. Nonetheless, there exist numerical differences in the gene expression levels. Hence, employing a combination of two genes as reference genes is deemed to be a more advantageous approach.

This study represents the initial exploration of appropriate internal reference genes for grain germination in quinoa, employing diverse exogenous hormone treatments. In essence, this research offers significant contributions to the understanding of seed dormancy and PHS, also paves the way for future investigations in the fields of molecular biology, genetics, and quinoa breeding.

CONCLUSIONS

Quinoa seeds were subjected to varying concentrations of ABA and GA, specifically 200 µM, 100 µM, 50 µM, and 10 µM. Notably, the concentrations of 100 µM ABA and 200 µM GA exhibited the most favorable outcomes.

Consequently, our findings recommend *ACT* as the internal reference gene for H_2O treatment, while the combination of *MON1* and *EIF3* is proposed for hormone treatment, as well as for experiments involving concurrent H_2O and ABA or GA treatments.

Improper selection of reference genes for quinoa seeds subjected to water treatment may lead to an overestimation of the expression level of the target gene. Conversely, when exposed to ABA and GA hormone treatments, such selection may result in an underestimation of the expression level of the target gene.

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