

Article

Validation of Reference Genes for Accurate RT-qPCR Normalization in *Aeluropus littoralis* Under Drought, Cold, and ABA Treatments

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Abstract

Halophyte plants, with genes responsive to abiotic stress, are promising candidates to enhance crop stress tolerance, but reliable RT-qPCR analysis requires the precise selection of candidate reference genes (CRGs) due to their inconsistent expression across tissues and stress conditions. In this study eight CRGs of *A. littoralis*, *AIEF1A*, *AIRPS3*, *ALGTFC*, *AIRPS12*, *AIUBQ2*, *AITUB6*, *ALACT7*, and *ALGAPDH1*, were analyzed to assess their stability for the normalization of RT-qPCR data under polyethylene glycol (PEG, 20% *w/v* for drought simulation), abscisic acid (ABA, 100 μ M), and cold stress (4 °C) treatments. The result of the algorithms suggested different CRGs for different treatments or tissue types. However the comprehensive analysis indicates that *AIEF1A* is the most stable CRG for PEG-treated leaf tissue, but *AITUB6* is preferable for PEG-treated root tissue, while for PEG-treated leaf and root tissues, *AIEF1A* can be suggested. For cold-stressed leaf and/or root samples, *AIRPS3* was the most stable. For ABA-treated leaf and root tissues, *ALGTFC* and *AIEF1A* were the most stable CRGs, respectively, whereas *AITUB6* was suggested for ABA-treated leaf and root tissues. Collectively, for all stresses combined (PEG, ABA, and cold), *ALGTFC* was the most stable CRG in leaf samples, while *AIRPS3* was the most stable in root samples and combined leaf and root samples. The validation analysis indicates a statistically significant difference (*p* value < 0.05) between normalization with the most and least stable CRGs. This research suggests reliable tissue-specific RGs for *A. littoralis* under abiotic stresses that can enhance the accuracy of gene expression quantification.

Keywords: abiotic stress; abscisic acid; polyethylene glycol; stable reference gene



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1. Introduction

Aeluropus littoralis, belonging to the Poaceae family, is a perennial monocotyledonous and halophytic grass with significant ecological and economic importance due to its remarkable tolerance to environmental stresses, potential for phytoremediation applications, and as a genetic resource for developing stress-tolerant crops [1,2]. Recent karyotyping on mitotic chromosomes revealed its chromosome number to be 20 ($2n = 2x = 20$), with evidence of extended nucleolus-organizing regions [1]. This halophyte can be found in several distinct geographical regions of Asia, Europe, and Africa [1]. *A. littoralis* can tolerate a high concentration of 1100 mM sodium chloride [3,4]. It has significance for agro-economic and plant breeding research.

By exploiting halophyte genetic resources, the understanding of tolerance mechanism to abiotic stress such as salinity and drought can be enhanced [5]. These plants not only

have great potential to reveal the mechanism of abiotic stress but also could be used as a valuable source of stress-responsive genes in plant breeding programs [3]. There are several instances of gene transfer from *A. littoralis* to other species with promising outcomes such as the ectopic expression of the *A. littoralis* plasma membrane protein gene in *Nicotiana tabacum* var. *Xanthi* which confers tolerance to salt, osmotic, H₂O₂, heat, and freezing stresses [6] and the transfer of the *ALSAP* gene to *Oryza sativa* 'Nipponbare' to decrease yield loss under drought conditions [7].

Over the past few years, due to advances in high-throughput sequencing technology, more genomes have been sequenced, and the expression of numerous genes involved in tolerance to abiotic stress has been studied [8]. As the genome sequence of *A. littoralis* is now available, it is more feasible to identify features of stress-responsive genes in the small genome of *A. littoralis* (~350 Mbp) [1,2]. To understand the mechanism of *A. littoralis*'s abiotic stress tolerance, it is necessary to use sensitive molecular methods such as RT-qPCR to study the expression of stress-responsive genes [9,10]. Key factors such as RNA quantity and quality, cDNA synthesis, PCR efficiency, and reliable normalization are vital for accurate mRNA expression analysis [11–13]. Normalization in expression studies corrects for technical variability to enable reliable relative analysis using internal control genes, commonly known as reference genes (RGs) or housekeeping genes (HKGs) [9,14].

HKGs such as actin (*ACT*), beta-tubulin (*TUB*), elongation factor (*EF*), polyubiquitin (*UBQ*), eukaryotic translation initiation factor (*eIF*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), histone (*HIS*), ribosomal protein S (*RPS*) or L (*RPL*), cyclophilin (*CYP*), and 18S ribosomal RNA (*18S rRNA*) are chosen as RGs for RT-qPCR normalization due to their consistent expression across diverse tissues, developmental stages, and stress conditions, as well as their critical roles in fundamental cellular processes [15–17]. However, recent RG studies indicate that RG selection is rather tissue- and experiment-specific, and therefore, the generalized usage of a single RG for the normalization of gene expression across different conditions should be avoided [11,13,18]. A literature review of halophytes reveals that while HKGs like *GAPDH* are favored for their presumed stable expression in RT-qPCR, their stability varies significantly across species, tissue types, and abiotic stresses like drought, salt, ABA, and cold. *GAPDH* is frequently identified as the most stable reference gene in *Salicornia europaea* under drought stress (not watered seedlings) [19] and *Suaeda aralocaspica* under different germination time points and salt stress conditions (300 mM NaCl) [20] but is notably unstable in *Hordeum brevisubulatum* under NaCl (350 mM) and ABA (20 µM) conditions [21], in *Rhizophora apiculata* tissues (leaf, root, stem, and flower) [22], as well as in *Suaeda glauca* at different salt concentrations (0, 50, 100, 150, and 200, and 400 mM NaCl) in different tissues (roots, stems, leaves, and shoots) and different growth stages (seedlings, adult plants, and senescent plants) [23] and in *Halostachys caspica* under salt (200 mM NaCl and 600 mM NaCl) and drought stresses (5% PEG6000 and 15% PEG6000), highlighting its stress-, tissue-, and species-specific variability and necessitating careful validation for reliable normalization.

The selection of suitable RGs typically comprises three steps: identifying candidate reference genes (CRGs), assessing their expression stability across diverse developmental/stress/tissue conditions, and employing the different algorithms/programs to determine the best RGs. Several algorithms/programs, including ΔC_t [24], BestKeeper [25], geNorm [26], NormFinder [11], and RefFinder [27,28], are commonly used to select the best CRG by calculating the stability values of these genes under different conditions [29]. BestKeeper, NormFinder, and geNorm determine RG stability by assessing the standard deviation (SD), correlation coefficient (*r*), inter- and intragroup expression variability, and M value with pairwise variation ($V_n/n + 1$), respectively [29]. RefFinder calculates the geomean of all stability weights from the geNorm, NormFinder, and BestKeeper results by

assigning an appropriate weight to an individual gene, which contributes to a comprehensive ranking of the most stable candidate genes [17,30].

Although a previous study identified ten candidate reference genes (CRGs) involved in various biological processes and molecular functions in *A. littoralis* under salt stress, proposing five sets of RGs and their optimal number for specific tissues (root and leaf) and conditions (salt-stressed and recovered plants) [15], the stability and reliability of these genes under diverse abiotic stresses remain underexplored. Given the significant spatiotemporal expression variations in CRGs in *A. littoralis* observed in previous studies, on the one hand, and with recent its genome sequencing [1,2] providing a foundation for advanced research, on the other hand, the evaluation of CRGs for identifying stable RGs to drought, cold, and other stresses has become inevitable to reliable gene expression studies in this halophyte. To date, no appropriate reference genes for drought, cold, or ABA in *A. littoralis* have been validated. Hence, we have selected eight common CRGs from previous studies [15], aiming to identify appropriate reference genes with stable expression in two different tissues of *A. littoralis* under polyethylene glycol (PEG), cold stress (4 °C), and abscisic acid (ABA) treatments. This study selected ABA treatment due to its role in regulating the expression of numerous ABA-dependent genes, which are influenced by various abiotic stresses, aiming to identify RGs with minimal sensitivity to ABA fluctuations. We further studied the relative gene expression of *AtHSP60.7* to compare and validate RG reliability in the RT-qPCR normalization step. Our findings improve the reliability of RT-qPCR results in *A. littoralis* by validating RGs, establishing a foundation for accurate gene expression studies under abiotic stresses in halophytes.

2. Materials and Methods

2.1. Plant Material

The seeds of *A. littoralis* (wild ecotype) were collected from natural populations in the Roddasht region, Isfahan Province, Iran (32°33'03.5" N 52°29'31.3" E). The sterilized seeds were cultured on the full-strength MS medium supplemented with 0.7% agar, vitamins, and 3% sucrose (pH 5.8). The growth chamber conditions were 25 ± 3 °C with a 16 h light/8 h dark photoperiod at a 100 µmol m⁻²s⁻¹ photon flux density using cool-white fluorescent light. The two-week-old seedlings were transferred to a hydroponic culture containing Hoagland's solution. To obtain identical plant material, cuttings from one *A. littoralis* mother plant were utilized, following the methodology of our previous study [15,31]. After one month, the seedlings were treated with polyethylene glycol (PEG) 20% as drought stress. Seedlings were subjected to cold stress in a refrigerated growth chamber (range ± 2 °C) set at 4 °C with a 16/8 h light/dark photoperiod for one week. Leaf and root samples were collected at time points including 0 (control), 3, 6, and 48 h post-stress (hps) and one week after treatments. The ABA treatment was performed at 100 µM by spraying the hormone solution on the leaves. Leaf and root samples were gathered at time points of 0 (control), 3, 6, 24, and 48 h post-stress (hps). The collected samples were stored at −80 °C for subsequent steps.

2.2. Total RNA Extraction

Total RNA was extracted using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and concentration of the RNA samples were checked by using a NanoDrop spectrophotometer (Biochrom WPA Biowave II, Biochrom Ltd., Cambridge, UK) at 260/280 nm absorbance. Further, the integrity and purity of RNA was determined by running the samples on a 1.2% agarose gel via electrophoresis.

2.3. cDNA Synthesis and RT-qPCR Analysis

RNA was reverse transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNase treatment (DNase I RNase-free, Thermo Scientific, Waltham, MA, USA) was performed to prevent gDNA contamination. qPCR was also conducted with three rDNA-based primers for the DNA contamination assay. The primer sequences and GenBank accession numbers of the related genes are presented in Table 1. The final cDNA products were diluted to a ratio of 1:10 and stored at -20°C for the RT-qPCR run.

Table 1. Candidate reference genes evaluated for expression stability in RT-qPCR normalization.

Gene Symbol	Accession No.	Gene Locus	Identity%	Description	Primer Sequence	Amplicon Size
ACT7	EE594539.1	AT5G09810.1	94.62	Member of the actin gene family	GTATGGCAACATCGTGCTCAG TGGAGCAACTACCTTAAT	118
EF1A	EE594715.1	AT1G07940	34	GTP binding the elongation factor Tu family protein;	TGCTGTCGGTGTCATCAA CTCCATCAAACGCCCTCATT	97
UBQ2	EE594598.1	AT2G36170	62	60S ribosomal protein L40-1	CTTGGTCTGCTGTTGTCTTG CACGGTTCACCTATCCATCAC	200
TUB6	EE594551.1	LOC117856123	73	Encodes beta-tubulin	TGCTGCCTGCTGTATCTT CGGAGGAACTTACTACTACATACT	109
GTFC	JZ191082.1	LOC123066744	77.89	General transcription factor 3C polypeptide 5-like	TTCCAAGTGGCCATCAGGTT AAAGGGCTTCTCTGCCTCTTG	108
RPS12	JZ191056.1	AT2G32060	64	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein	TTGGCAGACTCACGAAGG GATGGCGGATCAGGAGAC	147
GAPDH1	JN604531.1	AT3G04120	98	Encodes cytosolic GAPDH (C subunit) and involved in the glycolytic pathway	TGGGCAAGATTAAGATCGGAAT TTGATGTCGCTGTGCTTCCA	184
RPS3	JZ191044.1	AT5G35530	90	Ribosomal protein S3 family protein	ATTCACTGGCTGACCGGATG GTGCCAAGGGTTGTGAGGTC	107

RT-qPCR was performed on a CFX96 real-time PCR instrument (Bio-Rad, Hercules, CA, USA) with a total volume of 10 μL per reaction according to the company's protocol. The reaction mixture contained the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) with two-step cycling. After amplification, a thermal denaturing cycle with continuous fluorescence measurement was run from 55°C to 95°C to obtain dissociation curve analysis and to verify the primers' amplification specificity. All RT-qPCRs were carried out in three biological and three technical replicates. Curves were analyzed by CFX Manager (Bio-Rad) with a single threshold cycle and the subtracted curve fit method. At least one non-template control (NTC) was used for each primer pair's master mix. The amplification efficiency for each reaction was calculated by LinRegPCR [32]. Reaction efficiency for each primer pair was determined using serial dilutions of cDNA and standard curve analysis, following the methodology of our previous study [15]. All quantitative real-time PCRs were performed according to the MIQE guidelines [12].

For the assessment of the expression stability of the CRG across samples of the root and leaf, salt stress and recovery quantification cycle (C_q) values were analyzed using ΔC_t [24], geNorm [26], NormFinder [11], and BestKeeper [25]. In the ΔC_t method, the ΔC_t , standard deviation (SD) and average of the SD value of the CRGs were calculated. geNorm calculates the average pairwise variation (M value) between all tested genes, and lower M values indicate greater stability, and it also provides a pairwise variation (V value) to determine the optimal number of reference genes required for accurate normalization [26]. NormFinder

considers both intra- and inter-group variations to assign a stability value to each gene, with lower values representing higher stability [11]. BestKeeper analyzes raw Ct values to calculate the SD, the coefficient of variation (CV), and Pearson's correlation coefficients; genes with a low SD and a high correlation according to the BestKeeper index are considered the most stable [25]. For comprehensive ranking, the most stable reference genes obtained by four currently available computational programs (Δ CT, geNorm, NormFinder, and BestKeeper) were integrated by the RefFinder web-based tool [28]. Using rankings from individual programs, RefFinder assigns weights to CRGs and computes their geometric mean for the final ranking.

2.4. RG Reliability Test

The *AlHSP60.7* gene was selected based on bioinformatics analyses, and primers were designed using AlleleID software (version 7.5, Premier Biosoft, CA, USA). A taxon-specific/cross-species assay, based on aligned sequences of the *AlHSP* gene family, was performed to ensure specific detection of *AlHSP60.7*. Primers for *AlHSP60.7* were designed with sequences GGCAACCGAAGCGAATC (17 bp, T_m 62.9 °C, GC 58.8%) and TCTGTGTCCATAGCAGTGTT (20 bp, T_m 62.9 °C, GC 45%), yielding a 61 bp amplicon (annealing temperature 60 °C). Relative *AlHSP60.7* expression in *A. littoralis* leaf and root tissues was assessed under PEG, ABA, and cold stress to evaluate RG reliability for RT-qPCR normalization. Quantitative analysis of the data related to the relative expression level of the studied genes was done using the $2^{-\Delta\Delta CT}$ method [33]. Normalization was performed using the most and least stable RGs to evaluate accuracy. Expression levels were represented as log₁₀ fold change relative to control conditions. The data are means ($\pm$ SE) of three biological replicates, analyzed for significance ($p < 0.05$) using Duncan's multiple range test, with differences marked by unique letters.

3. Results

3.1. Primer Specificity Validation

Eight CRGs were selected to assess their stability for normalization in RT-qPCR data under PEG, ABA, and cold stress treatments. Table 1 provides the annotations of CRGs based on Arabidopsis and Gene Ontology Information Resources (TAIR, <https://www.arabidopsis.org>, accessed on 25 April 2025). The annealing temperature and specificity of the primer pairs were determined using the pooled cDNA of tissue samples, with each sample having equal quantities. The annealing temperature of the primers was raised to 60 °C, and a melt curve analysis was performed to verify their specificity. A single sharp peak was observed for primers with no primer dimer. The size of the amplicons associated with the CRG was also verified by agarose gel electrophoresis in 3 percent gels (Figure S1). For sample and primer validation, a fivefold diluted sample using the same pooled cDNA was used. The mean efficiency per amplicon obtained by the LinRegPCR software version 2014.8 was used in all gene expression calculation, following the methodology of our previous study [15].

3.2. C_q Value Analysis of CRG

The BestKeeper software (version 1) was utilized to evaluate all qPCR data gathered from eight CRGs (N = 80). The averages and standard deviation of the C_q values for each CRG were determined using C_q values from all experimental samples (Figure 1). The mean C_q values for the eight different PCR reactions in this investigation varied from 19.08 for *AlUBQ2* to 24.90 for *AlGTFC*. The mean C_q values of 19.08 and 19.24 for *AlUBQ2* and *AlTUB6*, respectively, indicated high transcript abundance for these two CRGs, while *AlACT7* and *AlGTFC* had mean C_q values of 24.30 and 24.90, respectively, indicative of the lowest transcript

abundance. The standard deviation (SD) of each Cq value was calculated separately for the PEG treatment in the root (N = 30) and leaf (N = 30), the ABA treatment in the root (N = 30) and leaf (N = 30), and cold stress in the root (N = 45) and leaf (N = 45). All samples (N = 80) were used to assess the difference between each Cq value and the mean. Each treatment of root and leaf tissues was assessed individually at 3, 6, 48, and 168 hpi for the treatment and control conditions. In total, *AIGAPDH1* exhibited the highest CV (2.50%), with the mean Ct value of 21.72, while *AITUB6* showed the lowest CV (1.34%) with the mean Cq value of 19.24. In general, *AIGAPDH1* indicated the min Cq value of 17.09 and the max Cq value of 33.10, which revealed the most unstable CRG. On the other hand, *AIGTFC* was the most stable CRG with the min and max Cq values of 22.53 and 28.32, respectively.

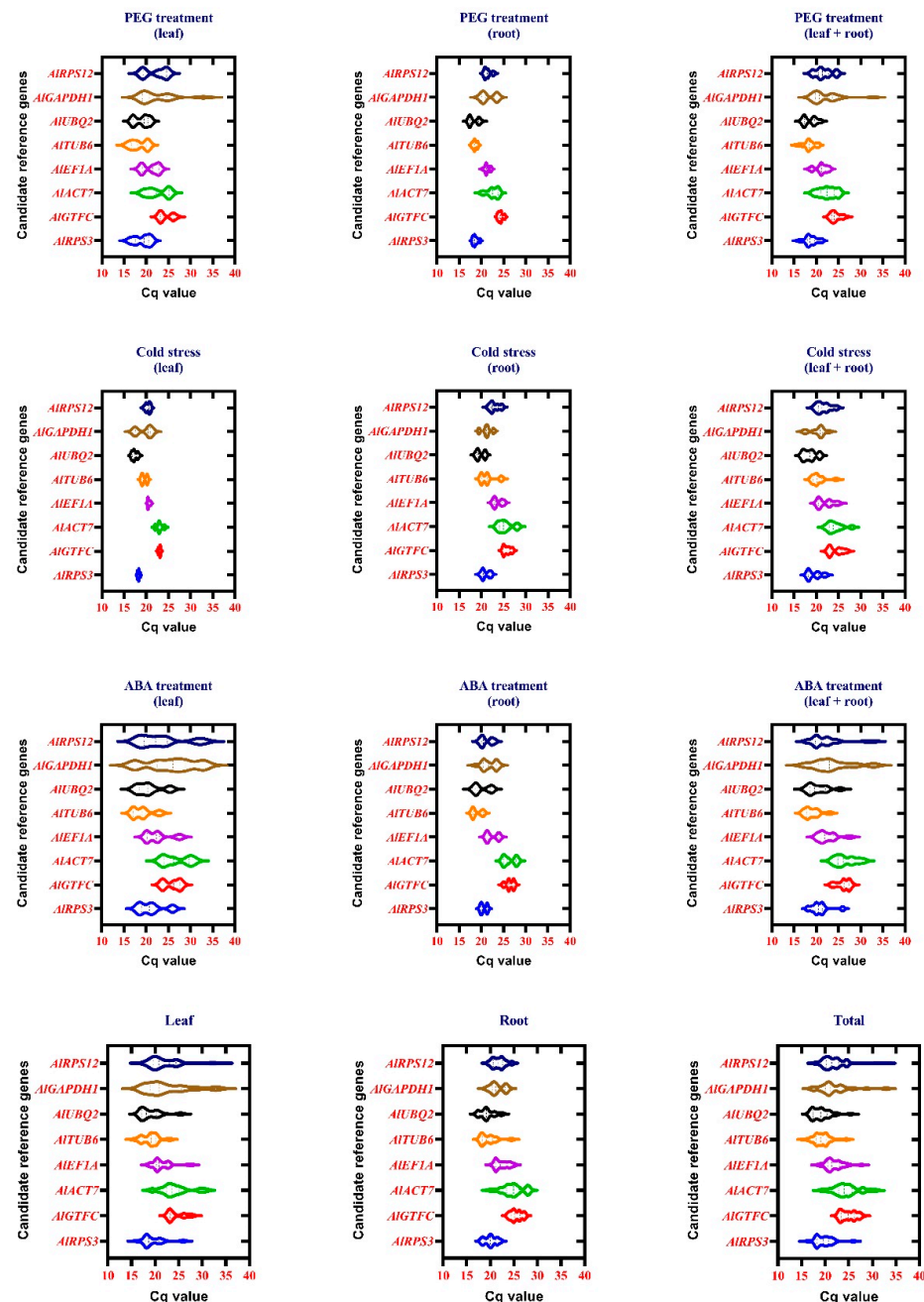


Figure 1. Violin plot representing the distribution of Cq values of *AIEF1A*, *AIRPS3*, *AIGTFC*, *AIRPS12*, *AIUBQ2*, *AITUB6*, *AIACT7*, and *AIGAPDH1* for PEG, ABA, and cold stresses in the leaf and root and combined tissues (leaf and root) of *Aeluropus littoralis*. The extension in horizontal length indicates higher instability in the expression of CRGs.

3.3. ΔC_t Analysis of CRG

The ΔC_t method was employed to compare the stability of CRGs in *A. littoralis* leaf and root tissues under PEG, cold, and ABA stress conditions (Figure S2). In PEG-treated leaf samples, *AIEF1A* was the most stable CRG with the lowest standard deviation (SD = 1), while *ALGAPDH1* showed the least stability (SD = 2.65). For PEG-treated root samples, *AIEF1A* was the most stable CRG (SD = 0.66), and *ALGAPDH1* was the least stable (SD = 1.22). For PEG-treated leaf and root samples, *AIEF1A* was the most stable CRG, while *ALGAPDH1* was the least stable. For cold-stressed leaf samples, *AIEF1A* was the most stable CRG (SD = 0.68), while *ALGAPDH1* was the least stable (SD = 1.77). For cold-stressed root samples, *AIRPS3* was identified as the most stable CRG (SD = 0.65), while *ALGAPDH1* was the least stable (SD = 1.29). For cold-stressed leaf and root, *AIRPS3* was the most stable CRG (SD = 0.71), and *ALGAPDH1* was the least stable (SD = 1.61). For ABA-treated leaf samples, *ALACT7* (SD = 2.21) was the most stable CRG, while *ALGAPDH1* was the least stable (SD = 4.68). For ABA-treated root samples, *AIEF1A* was the most stable (SD = 0.55), while *ALGAPDH1* was the least stable (SD = 0.94). For ABA-treated leaf and root samples, *ALACT7* (SD = 1.71) and *ALGAPDH1* (SD = 3.74) were the most and least stable CRGs, respectively. For leaf samples treated with all three stressors, namely PEG, ABA, and cold stresses (hereafter referred to as ‘total stress’), *ALGTFC* was the most stable CRG (SD = 1.80), while *ALGAPDH1* was the least stable (SD = 3.58). For root treated with the total stress, *AIEF1A* was the best CRG (SD = 1.01), and *ALGAPDH1* was the least stable (SD = 1.66). For the leaf and root treated with the total stress, *AIRPS3* was the most stable CRG (SD = 1.52), while *ALGAPDH1* was the least stable (SD = 2.89).

3.4. Bestkeeper Algorithm of CRG

The stability of CRGs was tested using the BestKeeper program by calculating the SD of the C_t values, correlation variation, and coefficient. Based on this analysis, the CRG with an SD value below 1.0 is suitable for gene expression normalization. In PEG-treated leaf samples, *ALGTFC* was the most stable CRG (SD = 1.41, CV = 5.78), while for PEG-treated root samples, *ALTUB6* was the most stable (SD = 0.38, CV = 2.05) (Figure S3). For leaf and root samples treated with PEG, *ALGTFC* was the most stable CRG (SD = 0.91, CV = 3.75). For cold-stressed leaf samples, *AIRPS3* was the most stable CRG (SD = 0.18), while *ALGTFC* was better for cold-stressed root samples (SD = 0.68, CV = 2.63), and *ALTUB6* was the most stable for the cold-stressed leaf and root (SD = 1.13, CV = 5.50). For ABA-treated leaf samples, *ALGTFC* (SD = 1.65, CV = 6.41) closely followed by *ALTUB6* (SD = 1.68, CV = 8.76) was the relatively more stable CRG. For ABA-treated root samples, *AIRPS3* was the most stable CRG (SD = 0.64, CV = 3.12), while *ALGTFC* was relatively better for ABA-treated leaf and root samples (SD = 1.17). For the leaf treated with the total stress, *ALTUB6* was the most stable CRG (SD = 1.34, CV = 7.06), while *ALGTFC* was better for the root treated with the total stress (SD = 1.0, CV = 3.91). For the leaf and root treated with the total stress, *ALTUB6* was relatively more stable (SD = 1.34, CV = 6.93).

3.5. GeNorm Analysis of CRG

The geNorm algorithm was used to compare the CRG in PEG-, cold-, and ABA-treated samples in the leaf and root tissues of *A. littoralis* (Figure 2). For PEG-treated leaf samples, the algorithm suggested *AIRPS3/ALTUB6* (M = 0.407), while in PEG-treated root samples, *ALGTFC/ALTUB6* (M = 0.317) were suggested. For the PEG-treated leaf and root, *AIEF1A/ALTUB6* (M = 0.474) were suggested. In cold-stressed leaf samples, *AIRPS3/AIEF1A* (M = 0.267) were suggested, while for cold-stressed root samples, *AIRPS3/RPS12* (M = 0.262) were suggested. For cold-stressed leaf and root samples, *AIRPS3/ALGTFC* (M = 0.324) were suggested. For ABA-treated leaf sample, the geNorm

algorithm suggested *ATUB6*/*ATUB6* ($M = 0.726$), while in the ABA-treated root sample, *AICT7*/*ATUB6* ($M = 0.289$) were suggested. For ABA-treated leaf and root samples, *ATUB6*/*ATUB6* ($M = 0.556$) were suggested. For the total stress in leaf samples, *ATUB6*/*ATUB6* ($M = 0.856$) were suggested, while for the total stress in root samples, *AICT7*/*ATUB6* ($M = 0.570$) were suggested. For the leaf and root treated with PEG, ABA, and cold stresses, *ATUB6*/*ATUB6* ($M = 0.919$) were suggested.

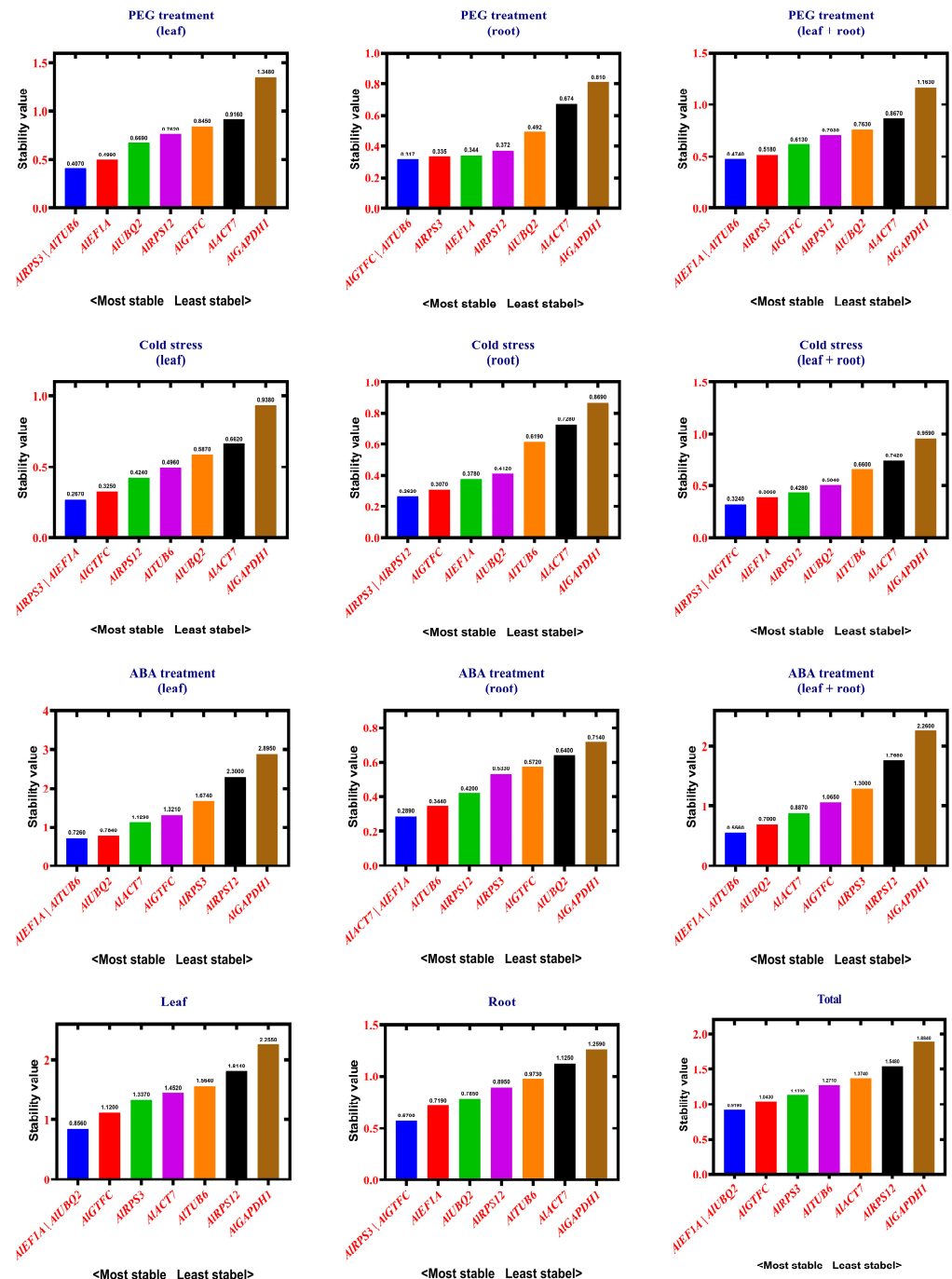


Figure 2. Profile of GeNorm algorithm for *AIEF1A*, *AIRPS3*, *AIGTFC*, *AIRPS12*, *AIUBQ2*, *AITUB6*, *AIACT7*, and *AIGAPDH1* for PEG, ABA, and cold stresses in the leaf and root and combined tissues (leaf and root) of *Aeluropus littoralis*.

3.6. Pairwise Variation Analysis

To determine the optimal number of CRGs for the normalization of each treatment and tissue type, pairwise variation (V_n/V_{n+1}) provided by the geNorm algorithm was

used between NFn and $NFn + 1$, with the stepwise inclusion of CRGs until the $Vn/Vn + 1$ ratio dropped below the recommended cutoff value of 0.15. For PEG-treated leaf samples, only the V6/7 value lowered the $Vn/Vn + 1$ ratio below 0.15 (0.134), while for PEG-treated root samples, only the V6/7 value was above the cutoff, and for PEG-treated leaf and root samples, 5/6 was the ideal combination (Figure 3). For cold-stressed leaf and/or root samples, the V2/3 value was below the cutoff. The least stability was estimated for the ABA-treated leaf sample with no $Vn/Vn + 1$ ratio below the cutoff, but for the ABA-treated root sample, the highest level of stability was observed with the V2/3 value. For the ABA-treated leaf and root, no $Vn/Vn + 1$ ratio was below the cutoff. In both tissues, no $Vn/Vn + 1$ ratio below the cutoff was found for the total stress.

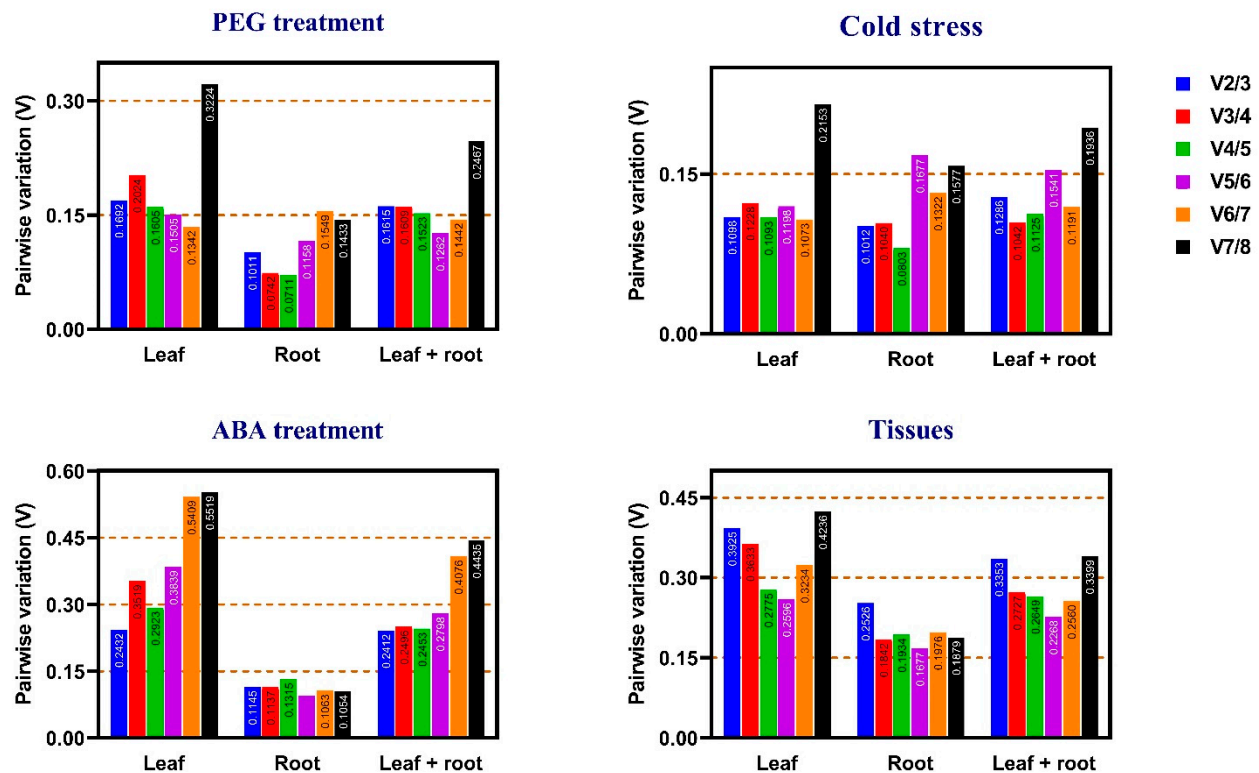


Figure 3. Pairwise variation provided by the geNorm algorithm for the ideal number of candidate reference genes (CRGs) for PEG, ABA, and cold stresses in the leaf and root and combined tissues (leaf + root) of *Aeluropus littoralis*. A $Vn/Vn + 1$ ratio below 0.15 is considered the ideal CRG combination.

3.7. Normfinder Algorithm of CRG

The Normfinder algorithm was used to compare CRGs in PEG-, cold-, and ABA-treated samples in the leaf and root tissues of *A. littoralis* (Figure 4). The algorithm selected *AIEF1A* ($M = 0.378$) closely followed by *AITUB6* for PEG-treated leaf samples, but for PEG-treated root samples, *AIRPS12* ($M = 0.355$), closely followed by *AIUBQ2* and *AIEF1A*, was suggested, while for PEG-treated leaf and root samples, *AIEF1A* ($M = 0.478$) was the most stable CRG. Based on the Normfinder algorithm, for the cold-stressed leaf sample, *AIRPS3* ($M = 0.133$) closely followed by *AIEF1A* was the most stable CRG, while for the cold-stressed root sample, *AIRPS12* ($M = 0.120$) closely followed by *AIRPS3* was the most stable. For the cold-stressed leaf and root sample, *AIRPS3* ($M = 0.081$) was the most stable CRG. For ABA-treated leaf samples, *AIAC77* ($M = 0.236$) was selected, while for the ABA-treated root, *AIEF1A* ($M = 0.119$) was selected. For ABA-treated leaf and root samples, *AIAC77* ($M = 0.298$) was the most stable CRG. For leaf stressed with the total stress, *AIRPS3* ($M = 0.629$) was the most stable CRG, while for the total stress in root samples, *AIEF1A*

($M = 0.388$) was the most stable. For leaf and roots treated with the total stress, *AIRPS3* ($M = 0.529$) was the most stable CRG.

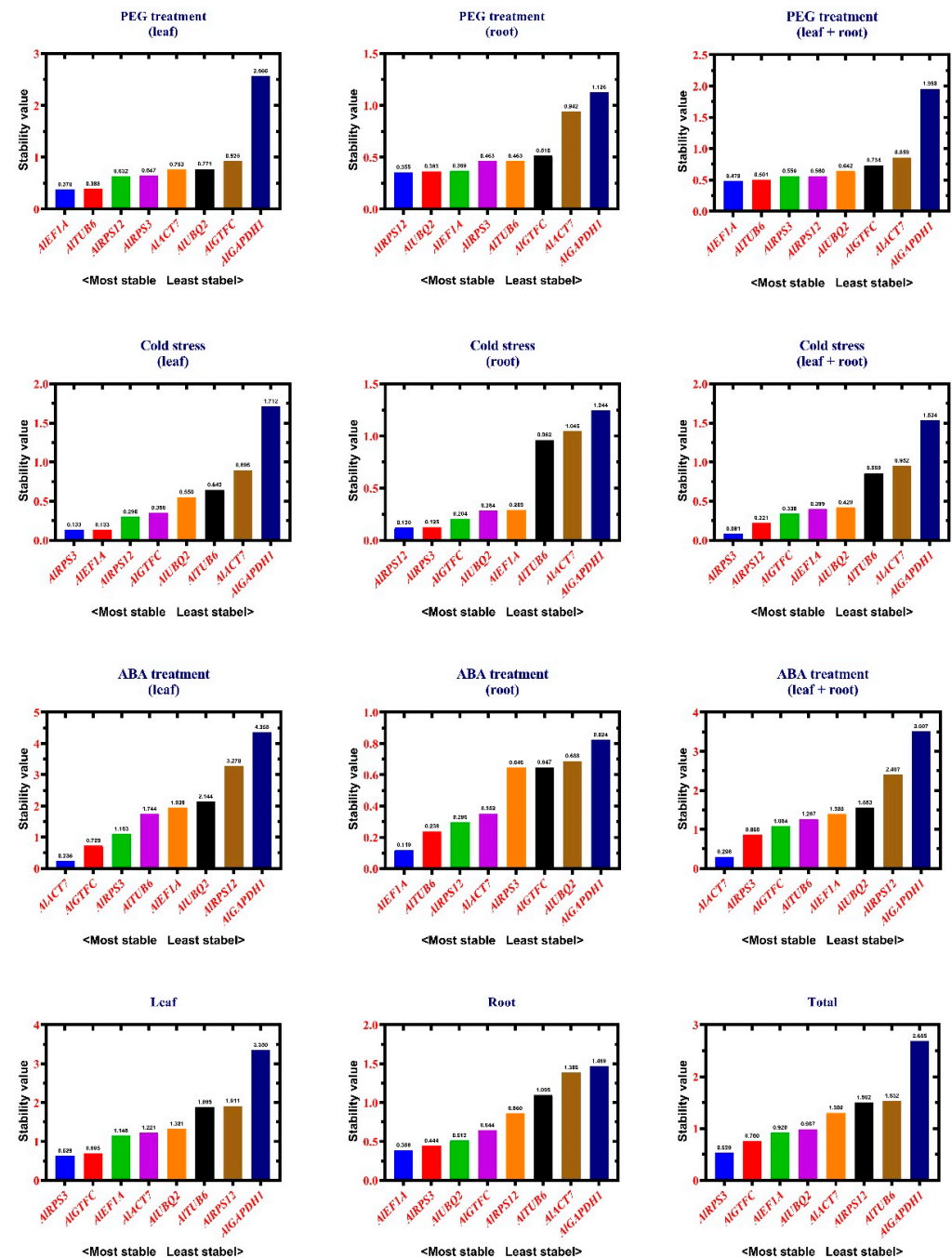


Figure 4. The result of the Normfinder algorithm for *AIEF1A*, *AIRPS3*, *AIGTFC*, *AIRPS12*, *AIUBQ2*, *AITUB6*, *AIACT7*, and *AIGAPDH1* for PEG, ABA, and cold stresses in the leaf and root and combined tissues (leaf and root) of *Aeluropus littoralis*.

3.8. Comprehensive Analysis of CRG

The comprehensive analysis provided by RefFinder integrated the result of several algorithms used based on the geometric means of the results. Based on the comprehensive analysis, CRGs were ranked for multiple conditions studied (Figure 5). For PEG-treated leaf samples, *AIEF1A* closely followed by *AITUB6* was suggested, while for PEG-treated root samples, *AITUB6* was more stable, but for PEG-treated leaf and root samples, *AIEF1A* was the most stable CRG. For cold-stressed leaf and/or root samples, *AIRPS3* was suggested.

For the ABA-treated leaf sample, *AlGTFC* followed by *AlACT7* and *AlTUB6* was suggested, but for the ABA-treated root sample, *AlEF1A* was suggested. For ABA-treated leaf and root samples, *AlTUB6* followed by *AlACT7* was suggested. For leaf treated with the total stresses, *AlGTFC* closely followed by *AlEF1A* was suggested, but for the root or leaf and root treated with the total stress, *AlRPS3* is suggested.

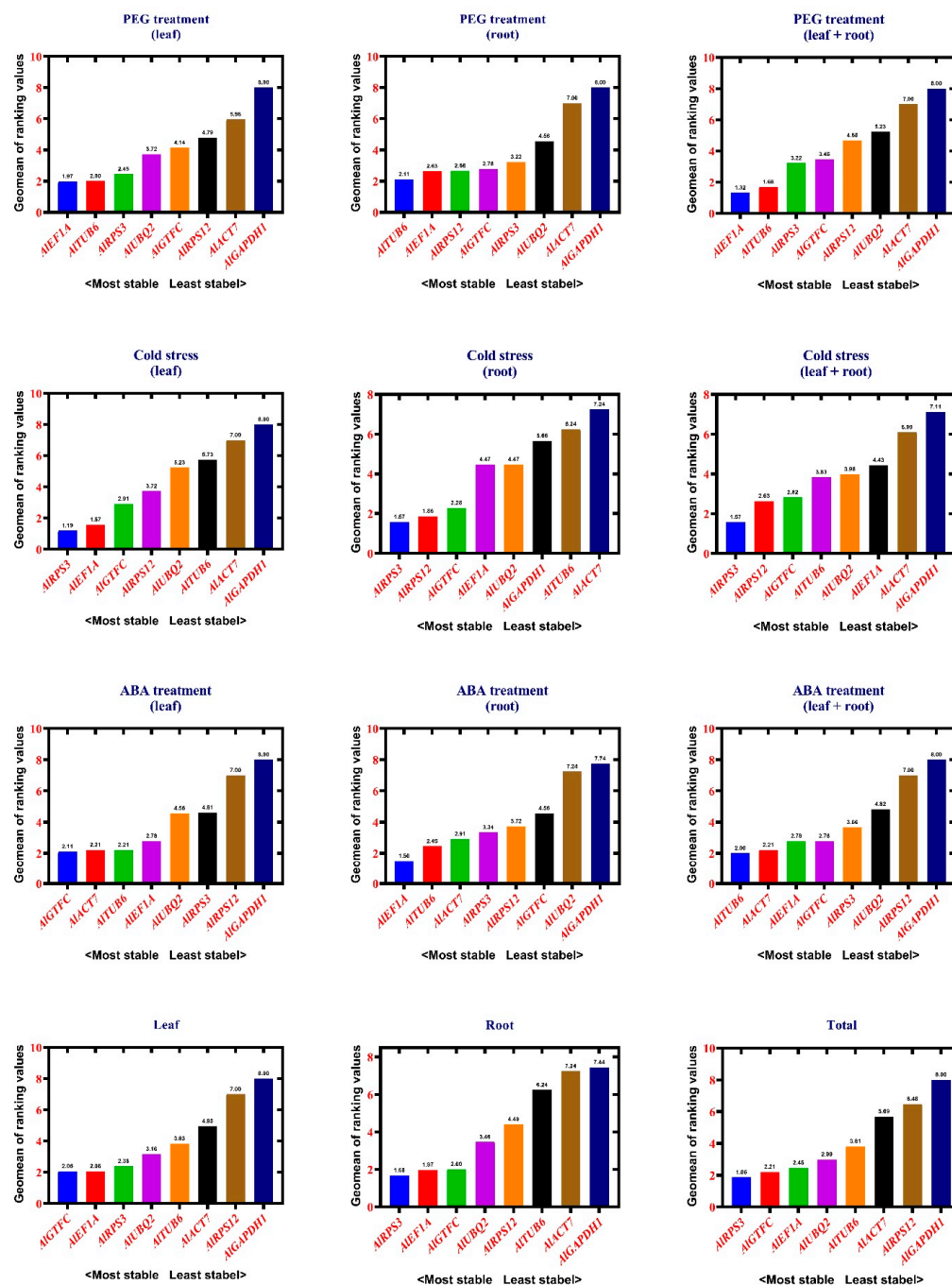


Figure 5. The result of the comprehensive analysis for *AlEF1A*, *AlRPS3*, *AlGTFC*, *AlRPS12*, *AlTUB6*, *AlACT7*, and *AlGAPDH1* for PEG, ABA, and cold stresses in the leaf and root and combined tissues (leaf and root) of *A. littoralis*.

3.9. Validation Analysis with Stable and Unstable CRGs

Different CRGs were used to normalize *AlHSP60.7* expression (log10 fold change) in RT-qPCRs (Figure 6). Samples were normalized with stable and unstable CRG for each treatment and the result separately were compared statistically (Duncan's multiple

range test, p value < 0.05). Pairwise comparisons of *AIHSP60.7* normalization with stable and unstable CRGs in PEG-treated *A. littoralis* leaf and root samples revealed significant differences at all time-points, except at 3 hps in leaf tissues. In *A. littoralis* cold-stressed leaf and root samples and ABA-treated leaf samples, pairwise comparisons of *AIHSP60.7* normalization using stable versus unstable CRGs showed significant differences at all time-points (3, 6, 48, and 168 hps). However, significant differences in *AIHSP60.7* normalization between stable and unstable CRGs were observed in ABA-treated root samples only at the 168 hps.

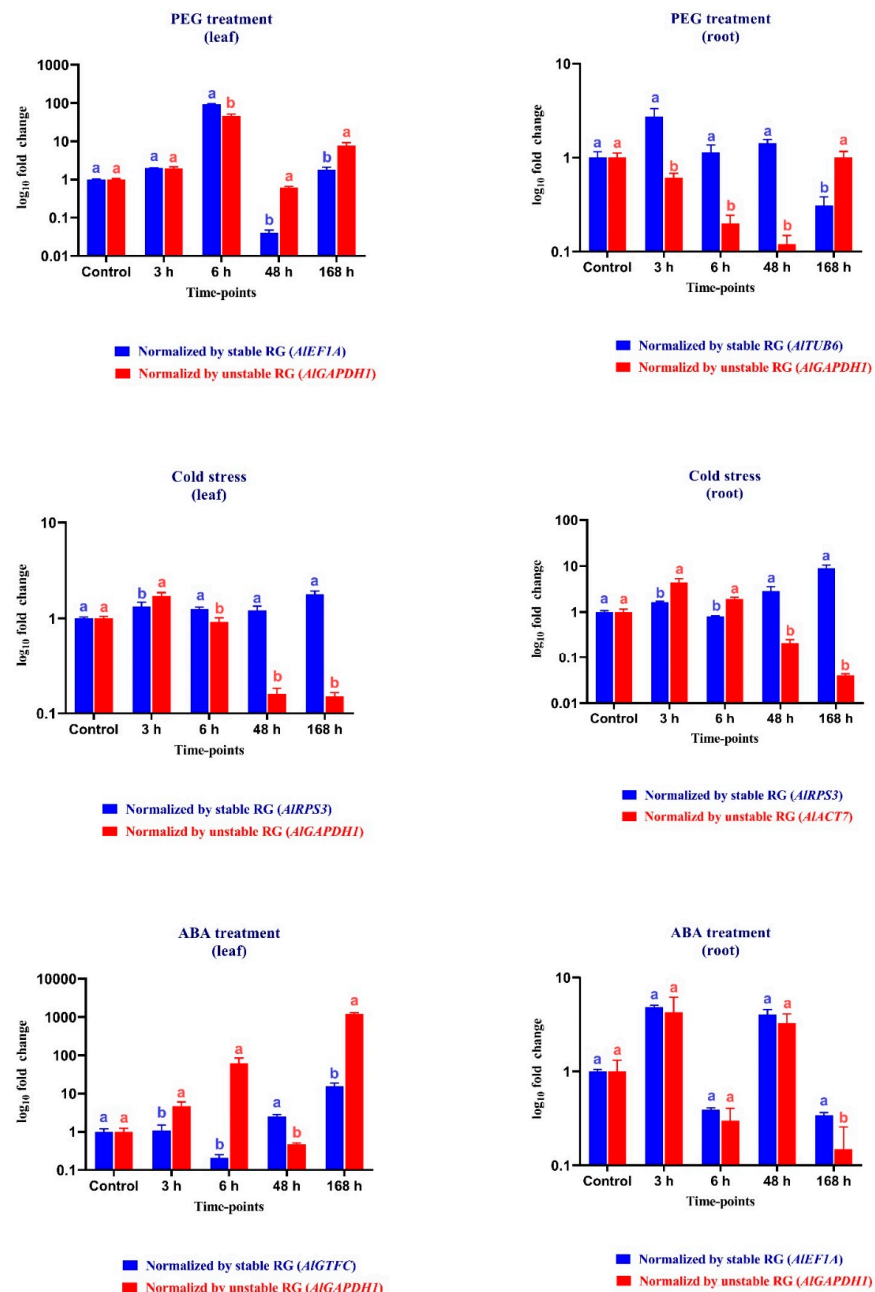


Figure 6. *AIHSP60.7* relative expression was used to compare and validate reference gene (RG) reliability in the RT-qPCR normalization step. *Aeluropus littoralis* *AIHSP60.7* gene expression was evaluated in leaf and root tissues under PEG, ABA, and cold stresses, normalized with the most stable (blue) and least stable (red) RGs. Expression levels are presented based on log₁₀ fold change stress/normal conditions. The values represent the mean ($\pm$ SE) of three biological replicates. Different letters above each bar indicate a significant difference ($p < 0.05$) based on Duncan's multiple range test.

4. Discussion

Abiotic stresses like drought, cold, salinity, and high temperatures are associated with pathways and genes involved [34,35]. Climate change is increasingly becoming one of the most widespread and devastating phenomena, which can increase the intensity of abiotic stresses. Although the outcome of climate change is complex and unpredictable, scientists are warning that the water crisis is on top of the consequences that can challenge all living kingdoms. Based on the IPCC Fifth Assessment report, big producers of staples are either on top or in the list of the most alarming sites in the world for water crisis, such as Southeast Asia [36]. Up to 30% drought-induced decline of wheat production is estimated for India, which is the second largest wheat producer, and even European countries such as France can be affected by this global threat [36,37]. To prepare for future food crises, one of the solutions can be exploiting the genetic resources of resistant plants for a plant breeding program. In line with this purpose, in this study, we examined the accuracy of gene expression analysis for future studies of *A. littoralis*, which is a halophyte model, and several reliable CRGs were introduced for corresponding conditions [6,7].

CRGs used here were GTP binding elongation factor 1-alpha (*EF1A*), ribosomal protein S3 family protein (*RPS3*), general transcription factor 3C polypeptide 5-like (*GTFC*), ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein (*RPS12*), 60S ribosomal protein L40-1 (*UBQ2*), beta-tubulin 6 (*TUB6*), *actin7* (*ACT7*), cytosolic *GAPDH* (C subunit) involved in the glycolytic pathway (*GAPDH1*) which were analyzed using several statistical algorithms to find suitable RGs in root and leaf of *A. littoralis* exposed to PEG, ABA, and cold stress. The ΔCt [24], NormFinder [11], geNorm [26], BestKeeper [25], and RefFinder [27,28] are reliable algorithms for finding stable CRG for RT-qPCR normalization. The pairwise comparison was used by geNorm for CRG ranking while the NormFinder and BestKeeper were used for further analysis of CRG. Similar to other studies, we detected inconsistency in result obtained by different methods used in this study. It is indicative of different nature of the calculation by the algorithm, however the result obtained by comprehensive analysis can be taken as the overall output. Overall, *AEF1A* was the best CRG for PEG-treated leaf. Using a common algorithm, *EF1A* was previously selected as the most stable CRG in other plants, such as Pitaya (*Hylocereus*) [38] and *Populus trichocarpa* for stability in temporal analysis [39]. However, for other monocotyledons, *EF1A* was ranked as the least stable RG in barley and oat infected with barley yellow dwarf virus (BYDV) [40]. Under salinity stress, *EF1A* was selected as the most stable CRG in soybean, while another CRG (*TUB4*) in drought-stressed soybean was selected as the most stably expressed internal gene. However, for other stresses such as dark and virus infection (SMV), neither of these two CRGs was acceptable for normalization [41]. It may indicate that each treatment requires an independent RG testing regardless of the common pathways involved [42]. In *Vigna mungo*, *EF1A*, *H2A*, and *ACT* were selected as the most stable CRGs for salinity stress [43]. Also, when stressed with yellow mosaic India virus, *EF1A* was still reliable for normalization of gene expression [43].

In our analysis, for normalization of gene expression in PEG-treated root, *ALTUB6* was finally suggested, while in *Vigna mungo* under drought stress, *TUB* was the most stable CRG [43]. However, in salinity or PEG-treated *Miscanthus sacchariflorus* *TUB* gene was not approved for gene expression normalization, in neither leaf nor in root tissue [44]. *TUB* was also the most stably expressed CRG in *Betula platyphylla* under osmotic stress [45]. Therefore, it might be suitable to include as a CRG for other abiotic stresses. *AIRPS3* was finally suggested for both tissue types in cold-stressed *A. littoralis*. These results are in agreement with our previous study in the root tissue of salt-stressed *A. littoralis* [15]. In another study, *RPS3* was recommended for normalization of gene expression in different tissues of *Cymbidium kanran* [46]. Interestingly, there are also reports indicating this gene as a stable CRG for gene expression in insects [47,48]. Also, our result showed that suitable CRG

varies significantly spatially when treated with PEG and ABA, whereas for cold-stressed *A. littoralis*, a similar CRG was suggested for all tissues and combined stresses. Although even for cold stress, when considering the output of each algorithm independently, the discrepancy emerges, which necessitates an all-inclusive-based decision.

For ABA the most stable CRG was different depending on tissue type. This result is also in agreement with the finding of ABA-treated *Glycyrrhiza* with different CRGs for leaf and root [49]. Translation initiation factor (*TIF1*) was the most stable CRG for their result in ABA-treated samples. The regulatory role of phytohormone ABA as a stress response regulator [50] can substantially affect HKGs stability which directly impacts reference gene selection during RT-qPCR normalization. Considering the combined stress (total stress) in leaf, *AlGTFC* was suggested, while for total stress in root or in leaf and root, *AlRPS3* was found to be most stable CRG. Ambroise et al. [51] reported that widely used *GAPDH* and β -*TUB* were the most unstable RG in several tissue types of *Salix viminalis* compared to newly introduced CRGs for RT-qPCR data normalization. These results are in agreement with our analysis, that the combination of CRG is better for the normalization of gene expression in plants challenged with several abiotic stresses. commonly used CRG, *GAPDH* was least stable for abiotic stress assessment in *Betula platyphylla* [45], which is in agreement with the result obtained in this study for *A. littoralis*. Li et al. concluded that *ACT* was the most stable RG in *Betula platyphylla* under different abiotic stresses, but when tissue types were considered, *ACT* was not recommended. *ACT* and *TEF* were the most stable RG for salt stress, while *TUB* was better for osmotic stress. Cold stress induced minimal Cq variability in *A. littoralis* leaf tissue compared to root across PEG, ABA, and cold treatments, contrasting with high Cq variation in salt-stressed roots from our prior study [15], suggesting salt stress imposes greater physiological constraints on roots (as a first line of defense) via ionic toxicity and osmotic imbalance, unlike cold stress's more uniform impact on tissues. In contrast to our prior study's high root Cq variability under salt stress, this study revealed lower Cq variability in leaf tissue than root, reflecting stress-specific physiological responses. Cold stress, notably at 48 and 168 h, showed the largest *AlHSP60.7* normalization differences between stable and unstable CRGs, despite minimal Cq variability, highlighting significant stability disparities.

Analysis of CRG across different tissues and stress conditions in *A. littoralis* resulted in accurate normalization of gene expression through the discovery of tissue-specific reference genes with condition-dependent characteristics. The unstable expression patterns indicate the necessity to choose RG through experimental validation for every experimental condition instead of applying universal RGs. The analysis proves that the commonly used reference gene, such as *GAPDH*, may introduce biases into expression analysis, which makes them unsuitable for generic internal normalization if not validated specifically. Our research revealed *RPS3* as one of the novel CRGs that show more expression stability in diverse tissue samples and experimental conditions compared to previous observations during salt stress analysis of *A. littoralis*. The results of this study showed that the evaluation process of RG for each specific tissue must be performed independently from other stress pathways. These findings demonstrate that ABA treatment needs a separate RG to study leaf and root tissues. Our complete examination demonstrated that CRG expression showed less variation under cold stress than PEG and ABA treatments, yet distinct reference genes should still be used for analyzing the same stress conditions across different plant tissues. The validation analysis shows statistically significant factors when normalizing data using the most stable reference genes compared to using the least stable reference genes. This demonstrates why selecting appropriate RG is essential for accurate results.

5. Conclusions

This study is the first to identify RG for precise gene expression normalization of *A. littoralis* under polyethylene glycol (PEG, 20% *w/v* for drought simulation), Absciscic acid (ABA, 100 μ M), and cold stress (4 °C) treatments. Using Δ Ct, geNorm, NormFinder, BestKeeper, and RefFinder, *AlEF1A* and *AlTUB6* were the most stable CRGs for PEG-treated leaf and root tissues in *A. littoralis*, *AlRPS3* for cold-stressed tissues, and *AlGTFC* and *AlEF1A* for ABA-treated leaf and root, respectively. In combined stresses (data pooled from PEG, ABA, and cold treatments) *AlGTFC* and *AlRPS3* were chosen for leaf and root, with *AlGAPDH1* being consistently unstable. The genetic resources of *A. littoralis* along with other halophyte species, are becoming more important because climate change can intensify drought and salinity stresses on global agriculture. This study also indicates that RG can be tissue and treatment-specific in *A. littoralis*, emphasizing the importance of validating RGs under specific experimental conditions to ensure accurate gene expression normalization. The result of this study is aligned with our previous findings, indicating that validation of RG through experiment-specific analysis is a prerequisite for reliable gene expression normalization.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy15071596/s1>, Figure S1. RT-qPCR products on a 3% agarose gel for eight candidate reference genes; Figure S2. The Δ Ct method was used to compare candidate RGs in PEG-, cold-, and ABA-stressed samples in the leaf and root tissues of *A. littoralis*; Figure S3. The result of the Bestkeeper algorithm on the stability of *AlEF1A*, *AlRPS3*, *AlGTFC*, *AlRPS12*, *AlUBQ2*, *AlTUB6*, *AlACT7*, and *AlGAPDH1*.

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Data Availability Statement: The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

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Abbreviations

The following abbreviations are used in this manuscript:

CRGs	Candidate Reference Genes
PEG	Polyethylene Glycol
ABA	Absciscic Acid
RG	Reference Gene
hps	Hours Post-Stress
NTC	Non-Template Control
Cq	Quantification Cycle

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