RESEARCH ARTICLE Legume Research- An International Journal, Volume 48 Issue 10: 1673-1685 (October 2025)

Screening of Internal Reference Genes Analysis in Alfalfa under Different Abiotic Stress Conditions

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10.18805/LRF-858

ABSTRACT

Background: Alfalfa is a kind of high-quality legume grass with high crude protein content, good palatability, high grass yield and good nitrogen fixation capacity. Abiotic stresses have become the key factors restricting the yield and quality in alfalfa and other crops, such as drought, extreme changes in ambient temperature, floods, land salinization and acidification, while under the different abiotic stress conditions, gene expression levels will also change accordingly. The selection and using of appropriate reference genes are crucial for the accuracy of gene expression quantification.

Methods: We chose the candidate internal reference genes of alfalfa from transcriptome sequence datasets (162 RNA-seq sequencing data) through comparative analysis. Finally, 10 candidate reference genes were selected. These candidate reference gene expressions were determined by RT-qPCR under three common abiotic stresses in production, such as alkali, drought and low temperature. The stability index of these candidate genes was calculated and evaluated correspondently using specific softwares and different algorithms, such as GeNorm, Normfinder, Bestkeeper, △Ct method and an online analysis tool RefFinder.

Result: The results showed that all 10 pair candidate reference genes could be used in gene expression quantification except for *GAPDH* and *Ms.33066* under low-temperature stress based on screening criteria. Under alkaline stress, the optimal reference gene is *UBL-2a* and the optimal combination of reference genes is *GAPDH* and *UBL*-2a; Under drought stress, the optimal reference gene is *Rer1* and the optimal combination of reference genes is *Ms.33066* (some candidate reference genes haven't been annotated yet, using gene ID abbre*via*tion number of *Medicago sativa* L. instead) and *Actin*; Under low-temperature stress, the optimal reference gene is *Ms.99505* and the optimal combination of reference genes is *Ms.65463, UBL-2a, Ms.99505* and *Actin*. Among all the samples, the optimal reference gene is *MS.99505* and the optimal combination of reference genes is *MS.073307*, *Rer*1, *MS.99505* and *UBL-2a*. GAPDH and Actin aren't the most appropriate reference genes of alfalfa under different abiotic stresses, the optimal reference gene and the optimal combination of reference genes under other abiotic stress need further validation. This paper provides scientific evidence for quantitative analysis of the genes of alfalfa.

Key words: Abiotic stress, Alfalfa, Reference gene, Screening.

INTRODUCTION

Alfalfa (*Medicago sativa* L.), is a kind of high-quality legume herbaceous with high crude protein content, good palatability, high grass yield and good nitrogen fixation capacity (Long and Zhang, 2024; Zhang and Long, 2024; Zhang *et al.,* 2023). It is well known for its high crude protein content and named the "King of Forage" (Sun *et al.,* 2019; Zhang *et al.,* 2015). The abiotic stresses have become constraints on alfalfa and other crops, affecting crop yield and quality, including drought and extreme changes in environmental temperature, floods, severe land salinization and acidification and other abiotic stresses (Elgharably and Benes, 2021; Fang *et al.,* 2022). Under abiotic stress conditions, gene expression levels also exhibit corresponding changes and appropriate or scientific reference genes are crucial for the scientificity and accuracy of gene expression quantification. Real-time fluorescence quantitative PCR can analyze the specific gene expression in alfalfa, such as changes in expression at different tissue sites, growth stages, or different environmental conditions (Gutierrez *et al.,* 2008; Udvardi *et al.,* 2008; Wan *et al.,* 2010). Compared with the traditional gene expression techniques of quantitative analysis, such as Northern blots, Western blots and cDNA microarrays, RT-qPCR (reverse transcription-quantitative PCR) is most widely used because of its low cost, time-saving and accuracy in gene expression (Dekkers *et al.,* 2012; Hao *et al.,* 2014; Li *et al.,* 2017; Ma *et al.,* 2016; Maroufi *et al.,* 2010; Tian *et al.,* 2015; Wu *et al.,* 2016; Zhu *et al.,* 2013).

To obtain more accurate and reliable results, it is usually necessary to introduce internal reference genes as reference corrections in RT-qPCR experiments to reduce the RNA quality and reverse transcription efficiency of different samples, as well as errors in the sample loading process and standardize the measurement of the target

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How to cite this article: Fan, W.N., Shi, P.F., Zhang, M.Y., Shi, Y.Q. and Yang, Y.X. (2025). Screening of Internal Reference Genes Analysis in Alfalfa under Different Abiotic Stress Conditions. *Legume Research*. **48(10):** 1673-1685. doi: 10.18805/LRF-858.

Submitted: 06-02-2025 **Accepted:** 08-07-2025 **Online:** 17-09-2025

gene. Reference genes, called housekeeping genes, described as "crucial for cellular existence" frequently, are often selected because of their high copy numbers and their stable expression level is rarely affected by the experimental conditions, or external environment (Joshi *et al.,* 2022; Kozera and Rapacz, 2013). Under an ideal situation, the selected reference genes should be expressed consistently in different growth and development stages, tissues and organs and different environmental conditions, without being affected by experimental conditions (Die *et al.,* 2010). The research results have indicated that there is no internal reference gene that fully meets the ideal criteria. Regardless of different developmental stages, environmental conditions, species, tissue sources, or experimental conditions, the expression of any reference gene will change and there is no universality between genes (Huggett *et al.,* 2005; Jain *et al.,* 2006; Winnepenninckx *et al.,* 1996). This phenomenon makes the selection of reference genes more complex.

The selection of reference genes requires certain conditions to meet, including expression stability, moderate abundance, independence from the target gene and external environmental influences and consistent expression within the cell. Selecting inappropriate housekeeping genes as reference genes can lead to significant biases and errors, resulting in inaccurate data. The selection and use of reference genes are key factors in ensuring more accurate and reliable experimental results (Small *et al.,* 1989; Wu *et al.,* 2021; Zhou *et al.,* 2016). A good reference gene for a qPCR experiment is one that is stably expressed in different conditions.

Currently, the study about the assembly of the genome of alfalfa is as follows: Chen *et al.* (2020) deciphered the genome of the cultivated alfalfa variety 'large leaf alfalfa' autotetraploid. Shen *et al.* (2020) deciphered the genome of the cultivated alfalfa variety 'Zhongmu No.1' autotetraploid. Long *et al.* (2022) deciphered the genome of the cultivated alfalfa variety 'Zhongmu No. 4' autotetraploid. Shi *et al.* (2024) provides a high-quality reference genome of an important diploid alfalfa germplasm. In production, the common alfalfa variety is tetraploid. We chose the large leaf alfalfa (cv.Xinjiang Daye) as a reference genome based on following justifications: Completeness and quality, large leaf alfalfa genome was the most complete and well-annotated genome available after comprehensive analysis. Utilizing a high-quality reference genome is critical for accurate mapping and subsequent analysis of RNA-seq data. Consistency across datasets: By mapping different RNA-seq data onto one single reference genome, we could get reliable gene expression atlas from multiple RNA-seq datasets. Conservation of reference genes: Reference genes which are the focus of our study are typically highly conserved across different cultivars of alfalfa.

In our study, the candidate reference genes of alfalfa from transcriptome sequence datasets (162 RNA-seq sequencing data through comparative analysis were chose, including different tissues of alfalfa). 10 candidate reference genes were selected. RT-qPCR was then adopted to determine the candidate reference genes under five abiotic stresses of drought, alkali and low temperature. The stability of these candidate genes was evaluated correspondently using specific software and algorithms, such as GeNorm (Vandesompele *et al.,* 2002), Normfinder (Andersen *et al.,* 2004), Bestkeeper (Pfaffl *et al.,* 2004), △Ct method (Silver *et al.,* 2006) and an online analysis tool RefFinder (Zsóri *et al.,* 2013). This research systematically explored the appropriate reference genes of alfalfa under different abiotic stresses to provide scientific evidence for quantitative analysis of the genes of alfalfa.

MATERIALS AND METHODS

Plant material

Three abiotic stresses (alkali, drought and low temperature) experiment were conducted using hydroponics (Ma *et al.,* 2021). Full and uniformly sized alfalfa seeds (Zhongmu NO. 3, from Beijing Institute of Animal Husbandry and Veterinary Medicine, Chinese Academy of Agricultural Sciences) were selected, soaked in $HgCl_2$ solution for 8 minutes and then rinsed repeatedly with distilled water to ensure there was no residue on the surface of the seeds. After soaking in distilled water for 12 hours, the soaked seeds were evenly spread in a culture dish with two layers of high-temperature sterilized filter paper. 40 seeds were placed on each dish, setting 3 repetitions. Then the culture dishes were placed in an artificial climate incubator for cultivation. The incubator simulated natural light conditions with three light intensity stages: 4000 lx, 6000 lx and full light in a day (light: darkness= 14 h/25°C:10h/20°C). Water the seedlings to keep sufficient moisture and conduct the treatments after cultivation for 14 days.

Alkali stress treatment

Add the Mixture of $NaHCO_3$:Na_2CO_3 (9:1) to the Hogland nutrient solution and set up four treatments: 0 mmol/L (CK), 30 mmol/L, 50 mmol/L and 70 mmol/L. Each dish is irrigated with 5 ml of treatment solution every morning and evening, with 5 replicates per treatment for 3 days.

Drought stress treatment

PEG-6000 was used to simulate drought stress using the weighting method. Irrigated different water potentials to ensure consistent weight per petri dish and weighed once every morning and evening. Set up four groups: control 0% (water potential, 0 MPa), mild drought 5% (water potential-0.10 MPa), moderate drought 10% (water potential-0.20 MPa) and severe drought 20% (water potential-0.40 MPa), 5 replicates per treatment for 3 days.

Low-temperature (Low T) stress treatment

Put the petri dishes in the artificial incubator for low-temperature stress treatment (under normal light conditions), The control group was set at 25°C, with the

low temperature set at 4°C and took samples every 4 hours (4h, 8h, 12h and 16 h), 5 replicates.

After the experimental treatment ended, we selected the 5 whole alfalfa normal seedlings randomly from each petri dish. Cleaned and dried with filter paper. Froze the samples with tin foil in liquid nitrogen immediately, then store them in a -80°C freezer.

RNA isolation and cDNA synthesis

Total RNA was extracted by RNAiso plus (Takara Co., LTD, Beijing, China) with RNA detection using NanoDrop 2000 Spectrophotometer and the reverse transcription reagent used the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Co., LTD, Beijing, China). All the reactions were conducted seriously according to the manufacturer's instructions.

Selection of candidate reference genes

We chose the candidate reference genes of alfalfa in transcriptome datasets (162 RNA seq sequencing data, the datasets generated and/or analyzed during the current study are available in the NCBI repository. The accession number and project number are provided in Attachment (Table 1) identify reference genes that can be stably expressed under different conditions and tissues, using 162 RNA seq sequencing data (including different tissues of alfalfa, root, stem, leaf and flowers). A total of 162 RNA-seq data from different alfalfa tissues, development stages and treatments were retrieved from NCBI SRA database. Then downloaded SRA file was first converted to FASTQ format. Sequencing adapters were removed using trim_galore (version 0.6.6, https://www.bioinformatics.babraham.ac.uk /projects/trim_galore/). Then sequencing data was converted to FASTA format using seqkit (version 0.14.0) (Shen *et al.,* 2016). All of the 162-sequencing data were then aligned to the alfalfa reference genome. The allele-aware chromosome-level genome assembly of this research was the most complete and well-annotated genome available, large leaf alfalfa (cv. Xinjiang Daye) using HISAT2 (version 2.2.1) (Kim *et al.,* 2019). Resulting SAM files were converted to BAM files and gene counts were obtained using featureCounts (version 2.0.1) (Liao *et al.,* 2014). Gene counts data was then fed into CustomSelection (version 1.0) R (version 4.1.2) package for candidate reference gene selection with the top genes cut-off of 0.05 (Santos *et al.,* 2020).

Primer design and RT-qPCR

Primers were designed using Primer Premier 5.0 based on the sequence retrieved from the 162 RNA-Seq datasets of alfalfa. Reactions were conducted using TB Green® Premix Ex Taq™ II (Tli RnaseHPlus) according to the manufacturer's instructions (Takara Co., LTD, Beijing, China) and amplified on a Bio-Rad Real-Time PCR system. The efficiency of 10 candidate reference gene primers was determined by the equation $E= [10^{(1/slope)} - 1] \times 100\%$ and the efficiency values should change between 90% and 110% (Radonić *et al.,* 2004).

Data analysis

The main methods for the results of RT-qPCR analysis of reference gene stability include programs, such as the △Ct method, GeNorm, Normfinder, Bestkeeper and an online analysis tool RefFinder. The ΔCt method calculates the SD value of each pair of candidate genes and the average SD value of each candidate reference gene to make comparisons. The GeNorm method calculates the candidate reference genes expression stability value (M value) and the pairwise variation value (Vn/n+1). Higher M values mean lower stability of gene expression. When the value of Vn/n+1 is less than 0.15, the reference gene has no significant contribution. NormFinder algorithm reveals the variation of candidate gene expression using the stability value (SV) when the internal reference genes for normalization by the ANOVA model. Lower SV means higher stability. The Bestkeeper method calculates and analyzes various internal reference genes by importing raw Ct values into an Excel spreadsheet. The stability is mainly analyzed using standard coefficient of variation (SD) and coefficient of correlation with variation (CV). The smaller the two values, the more stable the gene is. The RefFinder tool is a collection that combines the analysis and calculation of all the above algorithms. The results of the above analysis methods can be comprehensively evaluated and the conclusions can be seen more intuitively and clearly, avoiding the one-sidedness of using a single evaluation method to analyze genes.

RESULTS AND DISCUSSION

Although alfalfa has a high yield and good quality, making it the preferred forage for production and planting in feed crops, the research on the internal reference genes has not been conducted sufficiently for the normalization of gene expression. The reference gene selection requires certain conditions, including expression stability, moderate abundance, independence under external environmental influences and consistent expression within the cell. Currently, commonly used internal reference genes in alfalfa are *GAPDH* and *Actin* (β-actin) (Cui *et al.,* 2022; Li *et al.,* 2017; Ma *et al.,* 2016; Ma *et al.,* 2024; Wang *et al.,* 2023). In our study, the relatively more stable reference gene and reference gene combinations than *GAPDH* were found. With the development technique of RT-qPCR, reference genes' selection and validation for expression normalization were carried out. The candidate genes in alfalfa were retrieved from different tissues of alfalfa, root, stem, leaf and flowers (162 the transcriptome data of alfalfa).

RT-qPCR was always used to detect the reference genes' expression under the conditions of drought stress, alkaline stress and low-temperature stress and the stable reference genes were selected. To obtain the suitable reference genes, five calculation methods including GeNorm, Normfinder, Bestkeeper, △Ct and RefFinder were applied in analyzing the stability of these candidate reference genes in our work. The rankings in our results

Attachment Table 1: 162 RNA seq sequencing data.

Bio project accession number	Sequence read archive run number	Tissue	Reference
PRJNA276155	SRR1820204	Elongating stem	https://www.ncbi.nlm.nih.gov/bioproject/PRJNA276155
	SRR1820227	Elongating stem	
	SRR1820228	Elongating stem	
	SRR1820229	Flower	
	SRR1820230	Flower	
	SRR1820231	Flower	
	SRR1820232	Leaf	
	SRR1820234	Leaf	
	SRR1820235	Leaf	
	SRR1820328	Root	
	SRR1820330	Root	
	SRR1820332	Root	
	SRR1820677	Post elongating stem	
	SRR1820678	Post elongating stem	
	SRR1820691	Post elongating stem	
	SRR1820692	Nodule	
	SRR1820693	Nodule	
	SRR1820694	Nodule	
PRJNA414493	SRR6227642	Leaf	https://link.springer.com/article/10.1186/s12870-018-1250-4
	SRR6227643	Leaf	
	SRR6227644	Leaf	
	SRR6227645	Leaf	
	SRR6227646	Leaf	
	SRR6227647	Leaf	
	SRR6227648	Leaf	
	SRR6227649	Leaf	
	SRR6227650	Leaf	
	SRR6227651	Leaf	
	SRR6227652	Leaf	
	SRR6227653	Leaf	
PRJNA376067	SRR5279707	Root	https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA376067
	SRR5279708	Root	
	SRR5279709	Root	
	SRR5279710	Root	
	SRR5279711	Root	
PRJNA297135	SRR2529480	Whole seedling	https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA297135
	SRR2529481	Whole seedling	
	SRR2529482	Whole seedling	
PRJNA179114	SRR611809	Leaf	https://journals.plos.org/plosone/article?id=10.1371/journal.
	SRR611885	Leaf	pone.0122170
	SRR612113	Leaf	
	SRR612114	Leaf	
	SRR612115	Leaf	
	SRR612116	Leaf	
	SRR612163	Leaf	
	SRR612164	Leaf	
PRJNA558269	SRR9888362	Root	https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA558269
	SRR9888363	Root	

Table 1: Continue....

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	SRR9888364	Root	
	SRR9888365	Root	
	SRR9888366	Root	
	SRR9888367	Root	
PRJNA559760	SRR9949086	Leaf	https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA559760
	SRR9949087	Root	
	SRR9949088	Leaf	
	SRR9949089	Leaf	
	SRR9949090	Root	
	SRR9949091	Root	
	SRR9949092	Leaf	
	SRR9949093	Root	
PRJNA525327	SRR8667732	Root	https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA525327
	SRR8667733	Root	
	SRR8667734	Root	
	SRR8667735	Root	
	SRR8667736	Root	
	SRR8667737	Root	
	SRR8667738	Root	
	SRR8667739	Root	
	SRR8667740	Root	
	SRR8667741	Root	
	SRR8667742	Root	
	SRR8667743	Root	
PRJNA517930	SRR8507511	6^{th} Trifoliate leaves minus petiole	https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA517930
	SRR8507512	6^{th} Trifoliate leaves minus petiole	
	SRR8507513	6^{th} Trifoliate leaves minus petiole	
	SRR8507514	6^{th} Trifoliate leaves minus petiole	
PRJNA511802	SRR8381018	Root	https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA511802
	SRR8381019	Root	
	SRR8381020	Root	
	SRR8381021	Root	
	SRR8381022	Root	
	SRR8381023	Root	
	SRR8381024	Root	
	SRR8381025	Root	
	SRR8381026	Root	
	SRR8381027	Root	
	SRR8381028	Leaf	
	SRR8381029	Leaf	
	SRR8381030	Root	
	SRR8381031	Leaf	
	SRR8381032	Leaf	
	SRR8381033	Root	
	SRR8381034	Leaf	
	SRR8381035	Leaf	
	SRR8381036	Leaf	
	SRR8381037	Leaf	

Table 1: Continue....

Table 1: Continue....

	SRR8381038	Leaf	
PRJNA504525	SRR8173833	Root	https://link.springer.com/article/10.1007/s00344-020-10287-x
	SRR8173834	Root	
	SRR8173835	Root	
	SRR8173836	Root	
	SRR8173837	Root	
	SRR8173838	Root	
PRJNA489768	SRR8053193	Root	https://link.springer.com/article/10.1186/s12870-019-1773-3
	SRR8053194	Root	
	SRR8053195	Root	
	SRR8053196	Root	
PRJNA485535	SRR7685804	Cut Cotyledon_2	https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA485535
	SRR7685805	Cut Cotyledon 1	
	SRR7685806	Cut Cotyledon_3	
	SRR7685807	Embryogenic_2	
	SRR7685808	Cotyledon Embryo_1	
	SRR7685809	Embryogenic_1	
	SRR7685810	Embryogenic_3	
	SRR7685811	Non-Embryogenic_1	
	SRR7685812	Cotyledon Embryo_3	
	SRR7685813	Cotyledon Embryo_2	
	SRR7685818	Non-Embryogenic_3	
	SRR7685819	Non-Embryogenic_2	
PRJNA487676	SRR7751381	Root	https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA487676
	SRR7751382	Root	
	SRR7751383	Root	
	SRR7751384	Root	
	SRR7751385	Root	
	SRR7751386	Root	
PRJNA474585	SRR7262835	Leaf	https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA474585
	SRR7262836	Leaf	
	SRR7262837	Leaf	
	SRR7262838	Leaf	
	SRR7262839	Leaf	
	SRR7262858	Leaf	
	SRR7262859	Leaf	
	SRR7262860	Leaf	
PRJNA474427	SRR7252501	Leaf	https://www.mdpi.com/1422-0067/23/15/8633
	SRR7252502	Leaf	
	SRR7252503	Leaf	
	SRR7252504	Leaf	
	SRR7252505	Leaf	
	SRR7252506	Leaf	
	SRR7252507	Leaf	
	SRR7252508	Leaf	
	SRR7252509	Leaf	
PRJNA472434	SRR7193610	Leaf	https://link.springer.com/article/10.1186/s12864-018-5180-1
	SRR7193611	Leaf	
	SRR7193612	Leaf	
	SRR7193613	Leaf	
	SRR7193614	Leaf	
	SRR7193615	Leaf	

Table 1: Continue....

Table 1: Continue....

PRJNA602419	B80rep1	Flower bud	https://link.springer.com/article/10.1186/s12870-020-02775-9
	B80rep2	Flower bud	
	B80rep3	Flower bud	
	N195rep1	New leaf	
	N195rep2	New leaf	
	N195rep3	New leaf	
	N80rep2	New leaf	
	N80rep3	New leaf	
	O195rep1	Mature leaf	
	O195rep2	Mature leaf	
	O195rep3	Mature leaf	
	O80rep1	Mature leaf	
	O80rep2	Mature leaf	
	O80rep3	Mature leaf	

derived from the five methods were different slightly because using the different algorithms (Sabeh *et al.,* 2018).

By comparing five analysis software, △Ct method represents the genes' by comparing the candidate genes' average standard deviation values. The smaller the average standard de*via*tion value of a candidate reference gene, the more stable the genes' expression and the rule is the opposite conversely. GeNorm analyzes candidates based on their internal factors and sorts the similarity of expression in different samples. NormFinder calculates the stability value row sorting. BestKeeper directly pairs correlation analysis based on Ct values. If SD is more than 1, then this gene is directly excluded and it is considered to be the most unstable gene-expressed sample. It is usually used to enter preliminary screening, but GeNorm and NormFinder analysis methods are more effective. RefFinder is a comprehensive online tool, the analysis software combines the above four analysis methods (Andersen *et al.,* 2004; Pfaffl *et al.,* 2004; Silver *et al.,* 2006; Vandesompele *et al.,* 2002; Zsóri *et al.,* 2013).

Expression of candidate reference genes of alfalfa

10 candidate reference genes of alfalfa from transcriptome sequence datasets (162 RNA-seq sequencing data through comparative analysis were chose, including different tissues of alfalfa, Table 1). Primers of 10 candidate reference genes were used to amplify the cDNA template by PCR and all target amplicons obtained single strips, which were consistent with the expected target genes stripe size. All candidate genes' primers melting curves were plotted with a single peak, which showed the primers had no non-specific amplification with strong characters of specificity amplification. When the amplification length of the RT-qPCR product increased from 60 bp to 118 bp, these genes amplification efficiency changed from 90.55.3% to 105.07% (Table 1).

The Ct value analysis

The expression level is usually represented by Ct values, with smaller Ct values indicating higher gene expression levels and larger Ct values indicating lower gene expression levels. The Ct values fluctuation range reflects the genes' stability. The smaller the fluctuation range of Ct values, the more stable the genes are and the rule is opposite conversely. The analysis using the interquartile range of 10 candidate genes found that s, *Rer*1 gene has the highest expression abundance, while *MS.00617* gene has the lowest expression abundance; By comparing the interquartile range of 10 candidate reference genes, it can be seen that the fluctuation range of Ct values is in ascending order, *UBL-2a <MS.073307 <MS.65463 <Rer 1 <MS.99505 <MS.74923 <MS.00617 <Actin <MS.33066 <GAPDH* (Fig 1). However, there are outliers and extreme values in the *UBL-2a 0MS.073307*and *MS.65463* genes (Fig 1), only analysis of Ct values can't fully demonstrate the stability of the genes and further analysis is needed.

△ Ct method analysis

The △Ct method represents the genes' by comparing the candidate genes' average standard de*via*tion values. The smaller the average standard de*via*tion value of a candidate reference gene, the more stable the genes' expression and the rule is the opposite conversely. The result of △Ct analysis is shown in Table 2. Among the samples, *Ms.99505* showed the most stable performance with an average standard de*via*tion value of 0.99. Under alkaline stress, except for *Ms.74923*, the average standard de*via*tion values of other candidate reference genes were all greater than 0.99 and *Ms.99505* showed the most stable performance. Under drought stress, the average standard deviation values of the 10 candidate reference genes were all greater than 0.99 and *Rer 1* showed the most stable performance. Under low temperature stress, the average standard deviation values of *Actin* and *Ms.99505* were both greater than 0.99, indicating that *Actin* exhibited the most stable performance.

Norm finder analysis

Norm finder analysis calculates the S-value of reference genes, with the rule the more stable the gene, the smaller

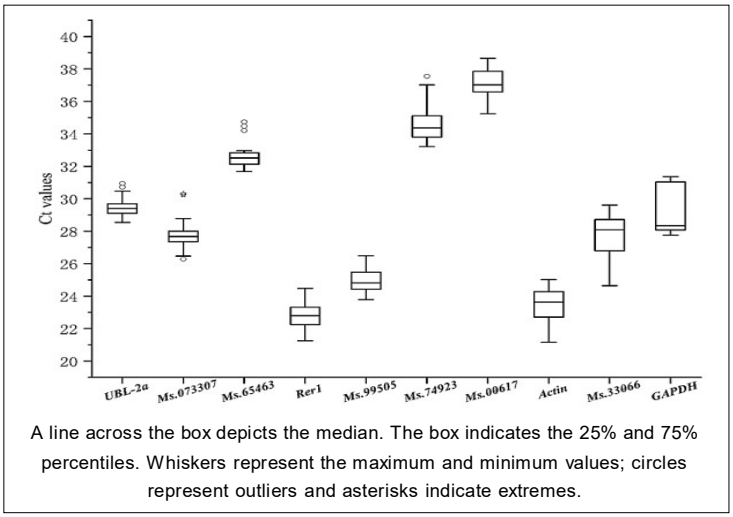
the S-value. Table 3 shows that under alkaline stress, *Ms.99505* exhibits the most stable performance; under drought stress, *Rer1* showed relatively stable performance; under low-temperature stress, *Actin* shows the most stable performance. Among all the samples, *Ms.99505* showed the most stable performance. The NormFinder analysis results in this study are basically consistent with those of △Ct method analysis.

Best keeper analysis

Best keeper measures the gene expression stability of these candidate reference genes by the Ct values of standard deviation and variation coefficient. Using SD=1 as the standard, reference genes with an SD value less than 1 are considered stable expressed genes. The smaller the value of the SD and CV are, the more stable the internal reference is and the rule is the opposite conversely. From Table 4, it can be seen that under alkaline stress, the standard de*via*tion of 10 candidate reference genes is less than 1 and *UBL-2a* shows the most stable performance; Under drought stress, the standard de*via*tion of all 10 candidate reference genes is less than 1, with *Ms.073307* showing the most stable performance; Under low temperature stress, except for *GAPDH* and *Ms.33066*, the standard deviations of the other 8 candidate reference genes were all less than 1,

Table 1: The primer sequences of candidate reference genes and the amplicon efficiency.

Gene symbol	Transcript_identifier/ Gene description	Primers	Amplicon length (bp)	TM (°C)	Efficiency (%)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	TTACAGTGAGGCTCGAGAAGGC CATCAACAGTCTTCTGGGTGGTTG	76	61	100.59%
Ms.74923	MS.gene74923.t1	CGGTGACAAAGATTGAGCACAGC AGAGCCTCCGGCGTAATTATCTG	66	59	100.75%
Ms.65463	MS.gene65463.t1	GAGATTAGAACGGCCTACAGTTGC TGAAGATGCGCTCCCAAAGCTC	71	59	99.17%
Ms.00617	MS.gene00617.t1	TTGTTGCCACTGGTGGGAAGTG TGGTTCAAACCGGAACGAAGGAG	79	60	94.13%
Ms.33066	MS.gene33066.t1	TTGAAACCGGTGACGCTACG AGCAAGTTCCAAACAGCTTCATCC	118	62	98.73%
Ms.073307	MS.gene073307.t1	CTGGTCCTGTTGGCAATAGTGG CGCTTCAAAGAGAACCACTGAGC	100	61	101.45%
Rer1	Endoplasmic reticulum sorting receptor 1	TCTGATGGTGGACCTTTGTTGCC TCAGGAAGCCGACGAATAAACGG	71	61	90.55%
Actin	Beta-Actin	TGTTGCTATTCAGGCCGTTCTTTC TCACCAGAATCCAACACAATACCG	72	60	96.57%
UBL-2a	Ubiquitin protein ligase 2a	TCATCCAAACAGCCAGATGACACC TCAGTCTCTTCCTTGCAGGAGTCG	74	62	105.07%
Ms.99505	MS.gene99505.t1	TTCCTCAGGTCTGCGAAGTAGG CAGTGGTGAAGAAACGCAAGACG	60	59	101.63%



A line across the box depicts the median. The box indicates the 25% and 75% percentiles. Whiskers represent the maximum and minimum values; circles represent outliers and asterisks indicate extremes.

Fig 1: Ct values for each reference genes in all samples.

with *Ms.99505* showing the most stable performance; Among all the samples, except for *GAPDH* and *Ms.33066*, the standard de*via*tions of the other 8 candidate reference genes were all less than 1, with *UBL-2a* showing the most stable performance.

All 10 pair candidate reference genes could be used in gene expression quantification except for *GAPDH* and *Ms.33066* under low-temperature stress based on screening criteria by the BestKeeper program.

GeNorm analysis

In the GeNorm method, the internal reference gene is determined by the sum of M values. The M value less than 1.5 indicates that it can be used as an internal reference gene and the lower the M value, the better the stability. The number of reference genes is determined by the relationship between the Vn/n+1 value and 0.15. Since the GeNorm method considers a single reference gene to be unstable, at least 2 reference genes are selected.

Table 2: The rank of RGs for normalization calculated by the ΔCt method.

Rank	Alkali		Drought		Low T		Total	
position	Gene	Average of STDEV	Gene	Average of STDEV	Gene	Average of STDEV	Gene	Average of STDEV
1	*Ms.99505*	0.59	*Rer1*	0.55	*Actin*	0.9	*Ms.99505*	0.99
2	*GAPDH*	0.6	*Ms.33066*	0.56	*Ms.99505*	0.93	*Rer1*	1
3	*UBL-2a*	0.61	*Ms.073307*	0.62	*Rer1*	0.99	*UBL-2a*	1.02
4	*Ms.00617*	0.61	*GAPDH*	0.63	*UBL-2a*	1.07	*Ms.073307*	1.09
5	*Ms.33066*	0.63	*Actin*	0.66	*Ms.65463*	1.1	*Ms.00617*	1.11
6	*Ms.65463*	0.63	*UBL-2a*	0.67	*Ms.073307*	1.12	*Actin*	1.14
7	*Rer1*	0.72	*Ms.74923*	0.69	*Ms.00617*	1.26	*Ms.65463*	1.14
8	*Actin*	0.77	*Ms.00617*	0.72	*GAPDH*	1.57	*GAPDH*	1.46
9	*Ms.073307*	0.92	*Ms.99505*	0.73	*Ms.33066*	1.61	*Ms.74923*	1.56
10	*Ms.74923*	1.25	*Ms.65463*	0.85	*Ms.74923*	1.64	*Ms.33066*	1.83

Table 3: The rank of RGs for normalization calculated by the NormFinder program.

Rank	Alkali		Drought		Low T		Total	
position	Gene	Stability value	Gene	Stability value	Gene	Stability value	Gene	Stability value
1	*Ms.99505*	0.21	*Rer1*	0.231	*Actin*	0.051	*Ms.99505*	0.394
2	*UBL-2a*	0.291	*Ms.33066*	0.271	*Ms.99505*	0.156	*UBL-2a*	0.456
3	*Ms.00617*	0.297	*GAPDH*	0.411	*Rer1*	0.473	*Rer1*	0.464
4	*Ms.65463*	0.317	*Ms.073307*	0.419	*UBL-2a*	0.615	*Ms.073307*	0.654
5	*Ms.33066*	0.32	*Actin*	0.463	*Ms.65463*	0.651	*Ms.00617*	0.66
6	*GAPDH*	0.324	*UBL-2a*	0.502	*Ms.073307*	0.705	*Ms.65463*	0.682
7	*Rer1*	0.497	*Ms.74923*	0.51	*Ms.00617*	0.975	*Actin*	0.696
8	*Actin*	0.568	*Ms.00617*	0.567	*GAPDH*	1.374	*GAPDH*	1.251
9	*Ms.073307*	0.836	*Ms.99505*	0.599	*Ms.33066*	1.444	*Ms.74923*	1.327
10	*Ms.74923*	1.199	*Ms.65463*	0.742	*Ms.74923*	1.446	*Ms.33066*	1.697

Table 4: The rank of RGs for normalization calculated by the BestKeeper program.

Rank	Alkali			Drought			Low T			Total		
position	Gene	Std. dev.	CV	Gene	Std. dev.	CV	Gene	Std. dev.	CV	Gene	Std. dev.	CV
1	*UBL-2a*	0.13	0.45	*Ms.073307*	0.16	0.58	*Ms.99505*	0.38	1.47	*UBL-2a*	0.38	1.3
2	*GAPDH*	0.14	0.51	*Rer1*	0.27	1.19	*Ms.65463*	0.41	1.27	*Ms.65463*	0.45	1.38
3	*Ms.65463*	0.25	0.78	*UBL-2a*	0.28	0.94	*UBL-2a*	0.53	1.78	*Ms.99505*	0.59	2.35
4	*Ms.00617*	0.26	0.7	*Ms.33066*	0.37	1.29	*Actin*	0.56	2.38	*Ms.073307*	0.6	2.14
5	*Rer1*	0.41	1.88	*Ms.74923*	0.39	1.15	*Rer1*	0.75	3.21	*Rer1*	0.66	2.9
6	*Ms.99505*	0.41	1.67	*GAPDH*	0.42	1.48	*Ms.073307*	0.87	3.05	*Ms.00617*	0.73	1.97
7	*Actin*	0.46	2.07	*Ms.99505*	0.42	1.7	*Ms.00617*	0.96	2.56	*Actin*	0.81	3.46
8	*Ms.33066*	0.52	1.87	*Actin*	0.61	2.52	*Ms.74923*	0.98	2.77	*Ms.74923*	0.92	2.66
9	*Ms.073307*	0.53	1.92	*Ms.00617*	0.68	1.83	*GAPDH*	1.06	3.46	*Ms.33066*	1.22	4.45
10	*Ms.74923*	0.95	2.73	*Ms.65463*	0.78	2.38	*Ms.33066*	1.09	4.16	*GAPDH*	1.25	4.29

Therefore, n in Vn/n+1 must be greater than or equal to 2. When V2/3<0.15, the 2 candidate reference genes with the smallest M value are selected as reference genes. If V2/3>0.15, a new reference gene is introduced. Compare the relationship between V3/4 and 0.15. If V3/4>0.15, another new reference gene is introduced until Vn/n+1<0.15 and the n candidate reference genes with the smallest M value are selected as reference genes.

From the GeNorm analysis results (Table 5 and Table 6), it can be seen that under alkaline, the M values of 10 candidate reference genes are all less than 1.5 and V2/3<0.15. *GAPDH* and *UBL-2a* with smaller M values are selected as reference genes; Under drought stress, the M values of 10 candidate internal reference genes are all less than 1.5 and V2/3<0.15. *Ms.33066* and *Actin* with smaller M values were selected as internal reference genes; Under low temperature stress, the M values of the 10 candidate internal reference genes were all less than 1.5 and V4/5 was less than 0.15. *Ms.65463*, *UBL-2a*, *Ms.99505* and *Actin* with smaller M values were selected as internal reference genes; Among all the samples, the M values of 10 candidate reference genes were less than 1.5 and V4/5 was less than 0.15. *Ms.073307*, *Rer1*, *Ms.99505* and *UBL-2a* with smaller M values were selected as reference genes.

Table 5: Average expression stability values (M) and ranking of the candidate RGs calculated using GeNorm.

Rank	Alkaline		Drought		Low T		Total	
position	Gene	M value	Gene	M value	Gene	M value	Gene	M value
1	*GAPDH*	0.255	*Ms.33066*	0.325	*Ms.65463*	0.454	*Ms.073307*	0.648
1	*UBL-2a*	0.255	*Actin*	0.325	*UBL-2a*	0.454	*Rer1*	0.648
3	*Ms.65463*	0.327	*Ms.99505*	0.393	*Ms.99505*	0.562	*Ms.99505*	0.708
4	*Ms.00617*	0.392	*UBL-2a*	0.478	*Actin*	0.665	*UBL-2a*	0.728
5	*Ms.99505*	0.441	*Ms.073307*	0.520	*Rer1*	0.715	*Ms.00617*	0.779
6	*Ms.33066*	0.463	*Rer1*	0.537	*Ms.073307*	0.768	*Actin*	0.829
7	*Rer1*	0.500	*GAPDH*	0.560	*Ms.00617*	0.845	*Ms.65463*	0.876
8	*Actin*	0.542	*Ms.74923*	0.588	*Ms.33066*	0.976	*GAPDH*	0.980
9	*Ms.073307*	0.602	*Ms.00617*	0.623	*GAPDH*	1.112	*Ms.74923*	1.086
10	*Ms.74923*	0.733	*Ms.65463*	0.669	*Ms.74923*	1.218	*Ms.33066*	1.235

Table 6: Pairwise varation to determine the optimal number of control genes for accurate normalization.

Alkaline		Drought		Low T		Total	
Pairwise variation	V value	Pairwise variation	V value	Pairwise variation	V value	Pairwise variation	V value
V2/3	0.113	V2/3	0.132	V2/3	0.192	V2/3	0.221
V3/4	0.104	V3/4	0.130	V3/4	0.178	V3/4	0.158
V4/5	0.091	V4/5	0.103	V4/5	0.148	V4/5	0.149
V5/6	0.076	V5/6	0.080	V5/6	0.128	V5/6	0.132
V6/7	0.072	V6/7	0.076	V6/7	0.134	V6/7	0.120
V7/8	0.073	V7/8	0.071	V7/8	0.157	V7/8	0.147
V8/9	0.083	V8/9	0.073	V8/9	0.166	V8/9	0.145
V9/10	0.121	V9/10	0.076	V9/10	0.147	V9/10	0.172

Table 7: The comprehensive ranking of RGs for normalization.

Rank	Alkali		Drought		Low T		Total	
position	Gene	Ranking values	Gene	Ranking values	Gene	Ranking values	Gene	Ranking values
1	*UBL-2a*	1.57	*Rer1*	1.86	*Ms.99505*	1.86	*Ms.99505*	1.73
2	*GAPDH*	2.21	*Ms.33066*	2	*Actin*	2	*UBL-2a*	2.21
3	*Ms.99505*	2.24	*Ms.073307*	2.78	*UBL-2a*	2.63	*Rer1*	2.34
4	*Ms.00617*	3.72	*Actin*	3.76	*Ms.65463*	2.66	*Ms.073307*	2.83
5	*Ms.65463*	3.83	*UBL-2a*	4.56	*Rer1*	3.87	*Ms.65463*	4.92
6	*Ms.33066*	5.89	*GAPDH*	4.92	*Ms.073307*	6	*Ms.00617*	5.23
7	*Rer1*	6.74	*Ms.99505*	6.18	*Ms.00617*	7	*Actin*	6.48
8	*Actin*	7.74	*Ms.74923*	6.65	*GAPDH*	8.49	*GAPDH*	8.46
9	*Ms.073307*	9	*Ms.00617*	8.49	*Ms.33066*	8.97	*Ms.74923*	8.74
10	*Ms.74923*	10	*Ms.65463*	10	*Ms.74923*	9.46	*Ms.33066*	9.74

RefFinder comprehensive analysis

RefFinder is a comprehensive analysis based on all the results of four methods: ΔCt, GeNorm, NormFinder and Best keeper. After integrating the ranking of candidate reference genes in different methods with certain weights, the ranking geometric mean is calculated. The lower the average value it is, the more stable it is; otherwise, it is unstable. From Table 7, it can be seen that under alkaline stress, the most stable internal reference gene is *UBL-2a*, has the highest un-stability index. Under drought stress, *Rer 1* has the highest stability. Under low-temperature stress, the most stable internal reference gene is *Ms.99505*; Among all the samples, the best stable reference gene is *Ms.9950*.

Under the full, complete consideration of the different ranks from the five algorithms, the rankings were calculated. Accordingly, the relative suitable reference genes or reference gene combinations were selected. *GAPDH* and *Actin* are taken as traditional reference genes commonly used in alfalfa. However, the results of our study showed that *GAPDH* were considered unstable reference genes. Though the *GAPDH* interquartile range is low, it has outliers. Based on the comprehensive validation of five methods analysis, the *GAPDH* doesn't have the highest stability. The reason why *GAPDH* is not the most stable and suitable internal reference gene may be because it not only serves as a component of the glycolysis pathway but also participates in other processes. Mallona *et al.* (2010) found that *GAPDH* is also not suitable for petunias and Dai *et al.* (2016) also proved that *GAPDH* does not have high stability in the late stage of grape development. Under different stress conditions in our study, *Actin* did not show good stability either, which is consistent with research results in plants such as *Arabidopsis* (Czechowski *et al.,* 2005), orchids (Zhang *et al.,* 2023), dogtooth roots (Chen *et al.,* 2015), soybeans (Luo *et al.,* 2023) and bamboo (Wu *et al.,* 2019).

There are certain differences in gene expression under different treatments, in different tissues, or also can be stably expressed under all changing conditions, including reference genes. Whether these optimal reference genes and the optimal combinations of reference genes are suitable under other abiotic stress is uncertainly. And these genes' stability also may be affected slightly by the different algorithms of the five-analysis software and the principles of gene screening (Andersen *et al.,* 2004; Hou, 2016; Kumar *et al.,* 2011; Pfaffl *et al.,* 2004; Silver *et al.,* 2006; Vandesompele *et al.,* 2002; Zsóri *et al.,* 2013).

CONCLUSION

This study provides the optimal single reference gene and combination of reference genes for alfalfa under different stresses. All 10 pair candidate reference genes could be used in gene expression quantification except for *GAPDH* and *Ms.33066* under low-temperature stress based on screening criteria. Under alkaline stress, the optimal reference gene is *UBL-2a* and the optimal combination of reference genes is *GAPDH* and *UBL*-2a; Under drought stress, the optimal reference gene is *Rer1* and the optimal combination of reference genes is *Ms.33066* and *Actin*; Under low-temperature stress, the optimal reference gene is *Ms.99505* and the optimal combination of reference genes is *Ms.65463, UBL-2a, Ms.99505* and *Actin*. Among all the samples, the optimal reference gene is *MS.99505* and the optimal combination of reference genes is *MS.073307*, *Rer1*, *MS.99505* and *UBL-2a*. *GAPDH* and *Actin* aren't the most appropriate reference genes of alfalfa under different abiotic stresses.

ACKNOWLEDGEMENT

This work was supported by the National Natural Science Foundation of China (32102585).

Conflict of interest

All authors declared that there is no conflict of interest.

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