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Reference gene selection for RT-qPCR normalization of strawberry at various organs, different growing stages of fruits, and salt-stress treatment

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Abstract: Strawberry (*Fragaria x ananassa* Duch.) is a favorite fruit of high economic value due to its good taste and high nutrition ingredients. Strawberry is a fruit grown around the world that has distinct genetic structures and indicates diverse levels of precision to different environmental circumstances. Plants respond differently to diverse physiological processes, organizing of biological events, control of hormones, different individuals of the same species, and internal and external factors in developmental stages. Quantitative real-time polymerase chain reaction (RT-qPCR) has become a very useful tool for the determination of plant genetic and physiological changes in gene expression. To obtain more securable gene expression outcomes, RT-qPCR data should be standardized with a control gene that shows homogeneous expression at diverse stages of growth in plants, in different organs, or under various environmental circumstances. We evaluated the gene expression of 8 reference genes, including StRefHISTH4, StRefGAPDH, StRefDBP, StRefActin1, UBQ, aTUB, 18SrRNA, and EF1a in different sets of 2 cultivars, four different organs, various fruit growing stages, and development period samples treated with salt. The genes expressions are greatly dissimilar in various organ samples examined. The expressions of StRefHISH4 and StRefActin 1 were very steady in all the different organs, fruits at various growth stages, and samples treated with salt analyzed. Furthermore, StRefHISH4 and StRefActin 1 showed the steadiest expression in plants cultivated under different development states. The expression of these reference (housekeeping) genes can be utilized for the standardization of real-time PCR outcomes for gene expression examination in many types of samples in strawberries.

Key words: Strawberry, real-time quantitative PCR, housekeeping genes, standardization

1. Introduction

Strawberry (*Fragaria x ananassa* Duch.) is a commercially significant fruit that is favored by receivers because of its good taste and nutrient substances (Hancock, 2020). The octoploid (2n = 8x = 56) strawberry has a matchless inherent evolutionary process that emerged as an interspecies crossbred of wild octoploid progenitor relatives about 300 years ago (Duchesne, 1976). Strawberry has a boosting growing field and rises in value due to its nice taste, aroma, basic components, minerals, vitamins, and antioxidant composites (Galli et al., 2015). Strawberry is a popular and important fruit in the Mediterranean diet due to its high content of essential nutrients and beneficial phytochemicals, both of which appear to have biological activity in human health (Gundesli et al., 2019; Okatan, 2020). Different ecological pressures can unfavorably influence plant development and fertility, cause structural change, chemical processes, and molecular alterations (Ali and Yun, 2017). Diverse physiological operations in plants are included in the stress reply, inclusive arrangement of biological membrane steadiness, organizing of hormone synthesis and backlog, and the efficiency of phenolic enzymes (Wilkinson and Davies, 2010). Different functional genes have major physiological roles in a series of actions at the molecular grade (Chen et al., 2012). Expression differences of tolerance genes to adverse conditions have helped the adaptation of plants to ecological circumstances (Zhang et al., 2016; Cao et al., 2020). Hereby, the recognition of physical environment stress-concerned genes in plants can provide beneficial knowledge regarding the molecular operations' basic reply to abiotic stress. Gene expression investigation can provide basic proof of gene activations in reply to exterior circumstance stress. Furthermore, quantitative real-time polymerase chain reaction (RT-qPCR) has been broadly utilized in gene expression research owing to its speed, sensitivity, correctness, and quantification (Gachon et al., 2004).

Transcriptomic experiments in every plant have stressed that the transcriptional organizing of complicated metabolic operations disclosed by these examines has a primary act (Shinozaki et al., 2018). However, the tasks of

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most of these genes have not yet been described (Liu et al., 2020). Transcript plenty in plants undergoing RT-qPCR is influenced by components such as reproducibility, RNA quality, elimination of dirty genomic DNA, correct reverse transcriptase reactions, plan of gene-special PCR markers, and choosing of the finest reference (housekeeping) genes for standardization (Udvardi et al., 2008). Accordingly, it is of major significance to choose a steady housekeeping gene for RT-qPCR standardization (Zhang et al., 2018; Liu et al., 2018). Therefore, investigating gene expression in varied plant species and under various ecological circumstances can provide useful insights into the molecular processes that will determine stress resistance and enable the advancement of genetic engineering. In addition, strawberry has become suggested as a model for genomic and transgenic activity investigated in the family Rosaceae (Mezzetti, 2009), so molecular information from this family can be applied to other species in this family.

Until now, one of the best techniques for defining gene expression is RT-qPCR because it is highly sensitive, repeatable, and clearly defining (Bustin, 2002; Derveaux et al., 2010, Galli et al., 2015: Zhang et al., 2018; Liu et al., 2020; Chen et al., 2021; Ye et al., 2021). However, the credibility of the outcomes of RT-qPCR depend on the feature of the RNA, its steadiness, and minimization of changes in the activity of the reverse transcription and PCR stages (Fleige and Pfaffl, 2006; Derveaux et al., 2010). In other words, an optimal reference gene must be at a stable expression grade under different circumstances and not be influenced by experimental situations (Liu et al., 2018).

Between these tactics, the choice of appropriate reference genes to standardize data is of major significance to acquiring the right outcomes. An appropriate housekeeping gene must be stated at a stable standard between materials, and its expression must not be influenced by the analysis circumstances (Bustin, 2002). The utilization of insufficient housekeeping genes can result in quantification mistakes; as a result, the expression information perhaps misinterprets (Jain et al., 2006; Amil-Ruiz et al., 2013). Reference genes play a role in the basic cellular events, fundamental metabolism, and protection of cell construction (Wong and Medrano, 2005; Czechowski et al., 2005). Therefore, the most conventional housekeeping genes now utilized in RT-qPCR researches in plants contain actin (ACT) (Maroufi et al., 2010; Zhang et al., 2018; Ye et al., 2021), tubulin (TUB) (Wan et al., 2010; Liu et al., 2020), ubiquitin (UBI) (Chen et al., 2011; Liu et al., 2020), 18S ribosomal RNA (18S) (Jain et al., 2006; Galli et al., 2015; Zhang et al., 2018; Ye et al., 2021), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Li et al., 2014; Liu et al., 2019; Galli et al., 2015; Zhang et al., 2018; Ye et al., 2021), histone H4 (HIS) (Galli et al., 2015; Zhang et al., 2018; Ye et al., 2021) and elongation factor 1-alpha (EF1α) (Liu et al., 2019). However, it is emphasized that the steadiness between some of these usually utilization reference genes is notional and not the only gene with steady and decided expression below whole experimental situations (Radonic et al., 2004; Czechowski et al., 2005). Hence, the trustworthiness of the conclusions of gene expression relies on the utilization of appropriate housekeeping genes. Housekeeping gene researches have been carried out in different plants like *Arabidopsis thaliana* (Czechowski et al., 2005), *Oryza sativa* (Kim et al., 2003; Ebadi Almas and Rahmani Kamrod, 2018), *Hypericum perforatum* (Zhou et al., 2019), *Puccinia triticina* (Prasad et al., 2020), *Lippia alba* (Lopes et al., 2021), *Agave sisalana* (Sarwar et al., 2020), *Saccharum officinarum* (Crystian et al., 2018), *Piper nigrum* L. (Umadevi et al., 2019) and Citrus (Keremane et al., 2021).

Many researchers straightly utilized genes included in cellular repair ways as reference genes for RT-qPCR analysis standardization without suitable reference gene choice, but the reference genes utilized might not be stable expression grades, particularly under dissimilar ecological circumstances. For this reason, researches to choose appropriate reference genes in distinct examples are essential to guarantee the right outcomes in RT-qPCR study. In past studies, some housekeeping genes from some plants were done with different outer applications and in diverse materials (Xiao et al., 2015; Lee et al., 2010). In the tea plant (*Camellia sinensis*), CsGAPDH displayed bad expression steady along with leaf growth and beneath hormonal procedures (Wu et al., 2016). FaACTIN and FaGAPDH2 were suggested as housekeeping genes for diverse tissues, pathogen effects, biotic stress, different fruit development, and senescence states in breed strawberries (Amil-Ruiz et al., 2013). In another study, FaDBP (DNA binding protein) was determined to be the best appropriate housekeeping gene to standardize gene expression in examples of two strawberry cultivars beneath water scarcity stress circumstances. The FaHISTH4 housekeeping gene had maximum expression steady at osmotic stress, while the FaGAPDH and Fa18S reference genes were determined the worst unstable genes (Crystian et al., 2018). Former studies of cultured strawberry and wild strawberry species identified some reference genes for standardization of gene expression in strawberries (Amil-Ruiz et al., 2013; Galli et al., 2015a; Yunting et al., 2018; Zhang et al., 2018, 2019; Ferreira et al., 2019; Liu et al., 2019; Chen et al., 2021). Ye et al. (2021) exposed strawberry (*Fragaria x ananassa*) seedlings to diverse abiotic ecologic circumstances and used seven nominee housekeeping genes. However, optimal housekeeping genes determined in cultured strawberries might not be used as suitable reference genes for wild strawberry species. In this study, we assessed the steadiest of four conventional

and four new candidate housekeeping genes, to define optimal housekeeping genes for expression stability in two strawberry cultivars (*Fragaria* × *ananassa* Duch), different organs, different fruit development periods and organs exposed to salinity. Four programs rely on diverse statistical algorithms were used to choose the most proper reference gene in the different samples. We evaluated eight independent experiments on seven samples in two strawberry cultivars. As a result, we determined the most suitable reference gene as an outcome of the study conducted with seven different samples and salt treatment in two strawberry cultivars.

2. Materials and methods

2.1. Plant growth conditions, NaCl treatments, and material collections

Different organs (root, stem, leaf, and fruit at different developmental stages) of strawberry cultivars "Camarosa" and "Rubygem" were harvested to confirm the expression of 8 housekeeping genes. "Camarosa" is a short-day (June) cultivar (U.S. Plant Pat. No. 5,262). However, it has higher yields, significantly earlier productivity, larger and more frequent fruiting, and a stronger structure. "Rubygem" is a medium to large fruited, early, sweet, high flavor and red colored road-hardy strawberry cultivar. It is a preferred cultivar in the domestic market and export. (Faostat, 2020). For this research, *Fragaria* x *ananassa* Duch. "Camarosa" and "Rubygem" cultivars grown in the Research and Experimental Area of Yaltır Tarım Company in Adana Province were used as plant material. In this research, the expression steady of used housekeeping genes was examined in the stated circumstances: (1) samples from two different cultivars; (2) samples from different organs; (3) fruits at different development stages; (4) fruits and tissue under salt stress at different development stages; (5) all samples.

For the application of salt stress, the plants were watered with 20 mMol/L sodium chloride (NaCl) every 2 days until mature fruit formation. Control plants were not treated with NaCl. Then, as shown in Figures 1a and 2a, for "Camarosa" and "Rubygem", different organs, leaves, stem, root, and fruit were collected at four different stages of development as plant materials. "Camarosa" fruits were harvested at four developmental stages: green (11 days after flowering, DAF), white (18 DAF), pink (28 DAF), and red (35 DAF) stages. "Rubygem" fruits were harvested at four developmental stages: green (13 days after flowering, DAF), white (21 DAF), pink (30 DAF), and red (38 DAF) stages. Leaves, roots, and stems were collected after every fruit harvest in the samples without salt application. In the salt-treated samples; leaves, roots, and stems were similarly collected after every fruit harvest. It was then placed in liquid nitrogen right away and kept at –80 °C until extraction and analysis. The essay occurred of a pitch on the design of three procedures: stress-free plants (control), plants exposed to salt stress, and organs at various growth stages. It comprises four repetitions with four plant replications for each material.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from gathered samples through the CTAB (hexadecyltrimethylammonium bromide) method (Carvalo et al., 2015). RNA quantity and quality were measured by 1% agarose gel electrophoresis; the rRNA bands were openly observable and defined that the RNA is unspoiled. RNA quality was also evaluated by identifying the OD 260/280 ratio utilization of a NanoDrop 1000 Spectrophotometer V3.7. Entire amounts were close to 2 showing fine RNA quality. Quantities were determined using the Qubit RNA BR assay kit (Invitrogen). Later, the RNA samples were diluted to 100 ng/µL. cDNA synthesis was applied utilizing the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific), and 5 µL of RNA was employed for cDNA synthesis. To identify the amplification efficiency (E) and correlation coefficient (®) analysis, the cDNA was diluted five-fold for controlling the qPCR analysis of all organs and treatments with NaCl. RT-qPCR reactions were applied to utilize the RealQ Plus master mixes (Amplicon) qPCR kit as proposed by the company. RT-qPCR reactions were realized using 3 µL of cDNA. Three detached tests (biological replicates) were applied for every gene and three copies (technical replicates) were utilized in every experiment.

2.3. Candidate reference genes selection and primer design

In this study, three reference primers from *F. vesca* genome sequences (https://www.ncbi.nlm.nih.gov), one reference primer from *F x ananassa* (Kurokura et al., 2006), and four frequently used reference primers were used (Liu et al., 2014; Zhu et al., 2013; Chang et al., 2012). According to the data in the table, primers have a melting temperature (Tm) of 49–65 °C and a GC content of 40%–55%. Eight candidate reference genes were chosen containing StRefHISTH4, StRefGAPDH, UBQ StRefDBP, StRefActin1, αTUB, 18SrRNA, and EF1α. Whole primer sequences and thematic data concerning the genes are available in Table 1.

2.4. RT–qPCR analysis

The attained cDNAs were diluted 5 times and qPCR reactions were applied. The qPCR reactions were carried out utilizing RealQ Plus 2X Master Mix Green (Ampliqon) as proposed by the company. qPCR analyses were applied with the Roche Lightcycler® 96 (Roche Life Science) device. Three microliters of cDNA was utilized in RT-qPCR reactions. The amplification program included 1

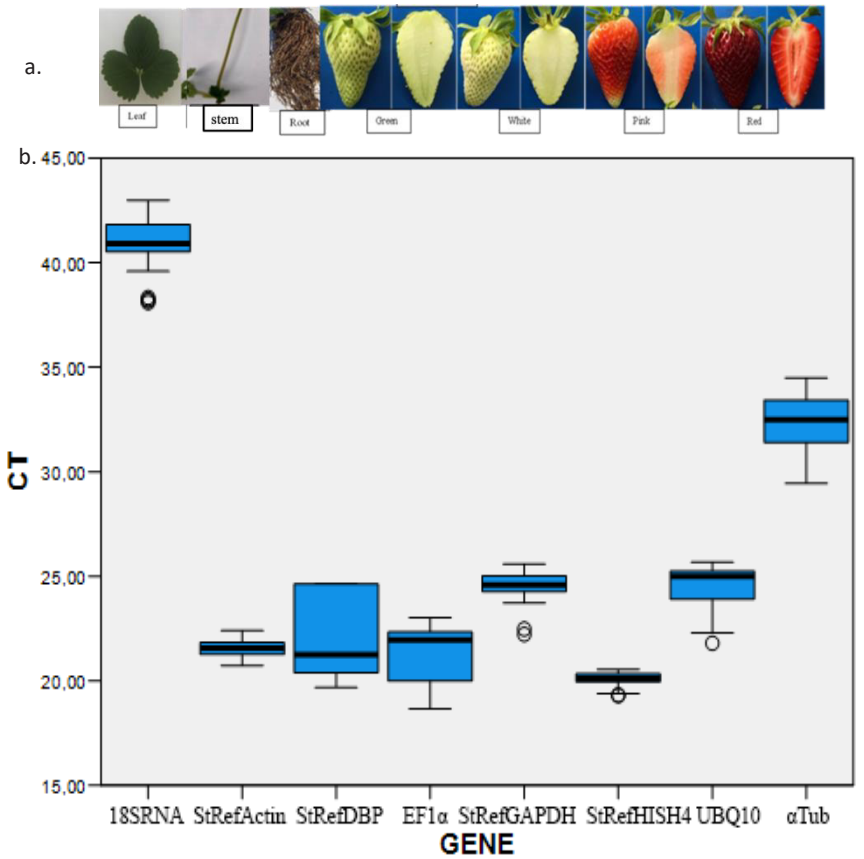


Figure 1. a. Sampling of ''Camarosa" strawberry cultivar (leaf, stem, root, green fruit, white fruit, pink fruit, red fruit). b. Ct values as a result of qRT-PCR acquired from 8 reference genes of ''Camarosa" cultivar, plants in different developmental stages, and all plants exposed to salt stress. Similarities or dissimilarities of Cq rates are shown as medians.

cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 min, 55 °C for 30 s, and 1 cycle of 37 °C for 30 s. The primers utilized in this analysis were prepensed by Universal ProbeFinder version 2.53 (https://lifescience.roche.com/en_tr/brands/universal-probe-library.html).

Average quantification cycle (Cq) rates of the ten-fold rarefaction cycle were created according to the logarithm of the combined cDNA dilution agents. The Cq rates and the watched equivalence were utilized to identify the efficiency (E) of every gene by the slope of a linear recession pattern: E %= (10[−1/slope] − 1) × 100% (Radonic et al., 2004). Amplification efficiencies were figured out of standard curves by adequate linear correlations (R^2> 0.99). Whole PCR periods showed efficiency from 90% to 110%.

2.5. Statistical analysis

The results of the qPCR data were obtained with Cq, and the Roche Lightcycler® 96 (Roche Life Science) data were transferred to a Microsoft Excel file. The efficiency of the PCR was predicted utilizing the LinReg PCR analysis (Ramakers et al., 2003). Four programs utilizing distinct algorithms, BestKeeper, geNorm, Delta Ct, and NormFinder, were used to evaluate the candidate housekeeping genes relying on expression steadiness calculations in the samples (Vandesompele et al., 2002; Andersen et al., 2004; Pfaffl et al., 2004; Silver et al., 2006). The ΔCt method determines the similarity or dissimilarity between the notional expressions of gene pairs in each sample to define advantageous housekeeping genes. The geNorm software calculated the mean expression stability rates (M) in the granting Cq datum of samples to evaluate the gene-expression stability. The genes with the lowest M values have the highest steadiness. NormFinder is an evaluation attempt utilized to supply the steady rate as a straight for assessment expression change. BestKeeper is an excel-based electronic document software that calculates ratios of gene-expression steadiness based on the standard deviation (SD) and coefficient of variance (CV) of Cq values. Sequencing of the candidate reference genes relies on their pair-wise relation with this sign rate, which is shown by the Pearson correlation coefficient (R). Normally, genes with upwards of expression steadiness will have down SD and CV values in BestKeeper analysis outcomes.

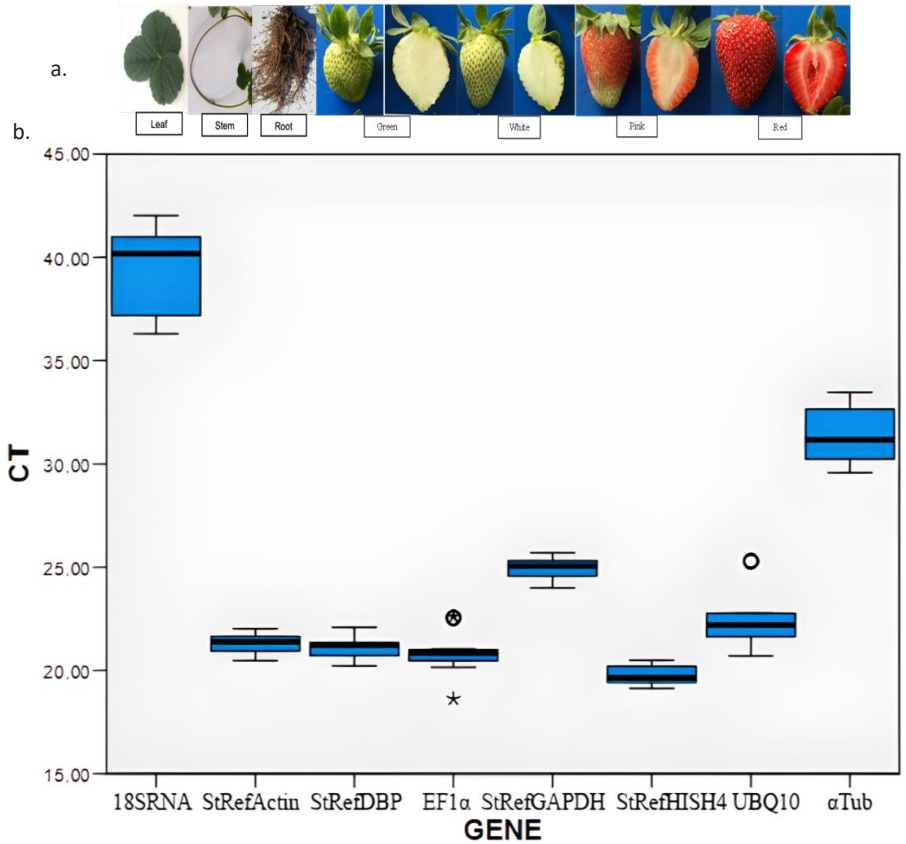


Figure 2. a. Sampling of ''Rubygem'' strawberry cultivar (leaf, stem, root, green fruit, white fruit, pink fruit, red fruit). b. Ct values as a result of RT-qPCR acquired from 8 reference genes of ''Rubygem'' cultivar, plants in different developmental stages and all plants exposed to salt stress. The box shows the 15th and 45th percentiles, and the horizontal lines symbolize the utmost and least rates. The up the boxes and stripes, the larger the discrepancy.

BestKeeper ranks the housekeeping genes concerning the SD and CV values (CV ± SD). Later, figuring out the pair-wise variation Vn/n+1, geNorm chooses the ideal number of check genes. The limit rate is generally adjusted to an accepted rate of 0.15. Gene expression stability and sequencing of 8 housekeeping genes were determined by geNorm using 23 serial samples. As a result of the analysis, StRefHISH4 and StRefActin 1 genes were determined as the most specific genes for ''Camarosa'' and ''Rubygem''. Moreover, we examined the sequences of housekeeping genes using the online RefFinder (http://www.leonxie.com/referencegene.php), a combination of the sequences generated by these four programs. Expression coefficients were attained according to the formula E−DDCT (Pfaffl, 2001).

3. Results

3.1. Expression profiling of housekeeping genes

As shown in Figures 1a and 2a, expression steadiness analyses of eight housekeeping genes were appraised with RT-qPCR in ''Camarosa'' and ''Rubygem'' to define the best stable reference gene at distinct growth phases, distinct organs under salt stress, and distinct growth phase of fruits, and the primers are given in Table 1. Based on RT-qPCR experiment results, the mean Cq ratios of the eight housekeeping genes of ''Camarosa'' ranged from 18.18 (EF1α) to 44.89 (18SRNA), while the Cq ratios of Rubygem ranged from 18.19 (EF1α) to 43.61 (18SRNA). As shown in Tables 2 and 3, standard deviation (SD) values were 0.0–1.97 in ''Camarosa'' and 0.28–4.02 in ''Rubygem''.

High RT-qPCR efficiency is generally correlated with strong and exact outcomes of gene expression. In this research, the efficiency of RT-qPCR was figured out for all candidate reference genes as the average rates acquired from the practice and biological repeats and it differs from 1.87 (UBQ) to 2.00 (EF1α), showing high efficiency. While Tm ranges from 49.57 ℃ (StRefGAPDH) to 72.17 ℃ (αTUB) across the overall PCR efficiency, this ratio is based on the predicted CG ratio and content, and is used as a measure in primer design (Bustin et al., 2009).

Table 1. List of 8 candidate reference genes tested.

Gene symbol	Primer sequence (5′-3′)	(Tm (°C)	Gene/protein ID	Gene name	R^2	RT-qPCR efficiency[a]
StRefGAPDH	GAGTCTACTGGAGTGTTCA	50.3	LOC101307033	Glyceraldehyde-3-phosphate	0.998	1.98
	CCTGTATTCGTGCTCATTCA	49.57				
StRefActin 1	TTCACGAGACCACCTATAACTC	53.35	LOC101300025	Actin 1	0.999	1.99
	GCTCATCCTATCAGCGATT	51.53				
StRefDBP	TTGGCAGCGGGACTTTACC	58.46	LOC101312116	DNA binding protein	0.997	1.99
	CGGTTGTGTGACGCTGTCAT	58.49				
StRefHISH4	TCAAGCGTATCTCCGGTCTC	56.57	AB197150.1	Histone	0.999	1.99
	AGT GTC CTT CCC TGC CTC TT	58.54		H4		
18s RNA	TTCACACCAAGTATCGCATTTC	67.59	X15590.1	18S ribosomal RNA	1.000	1.87
	CCAAGGAAATCAAACTGAACTG	67.59				
EF1a	AGATGGTTCCCACTAAGCCTATG	71.76	JX272638	Elongation factor	0.999	2.00
	ACACTCTTGATGACTCCAACTGC	71.76		1-alpha		
aTUB	CCACATCTCTTAGGTTTGATGGAG	72.17	LOC832097	Alfa-tubulin	0.999	1.99
	GGGTCACACTTGGCCATCAT	70.3				
UBQ	AGGGGAGGCATGCAGATTTT	68.25	LOC832184	Ubiquitin-conjugating enzyme	1.000	1.87
	AGGAATGCCCTCCTTGTCCT	70.3				

We can see from the box graphs in Figures 1b and 2b that the expression level of the 18SRNA gene was unstable in eight candidate reference genes among which the StRefHIS4 and StRefACT 1 gene regions had the lowest changeable Cq values.

3.2. Expression stability analysis by RefFinder programs

3.2.1. geNorm analysis

The decisiveness of the housekeeping genes in organs at different developmental stages and in cultivars with salt treatment was determined with the M rates by geNorm analysis. The M rate is described as the average distinction of a specific gene in reference genes. Housekeeping genes with the lowest M values show the highest expression steadiness (Umadevi et al., 2019). The studied gene has acceptable expression steadiness if the M rate is less than 0.15 (Allen et al., 2008; Gutierrez et al., 2008). The M rates of the eight reference genes were below 0.15 with regard to expression steadiness in different development stage organs and under salt treatment (different tissue, different fruit development periods, plant tissues exposed to salinity, and all samples in ''Camarosa" and ''Rubygem", as shown in Figures 3 and 4, respectively). The StRefHISH4 and StRefActin 1 were the genes that showed the most trustworthy expressions and had low M values in the course of fruit growth, the M rates of 4 genes were under 0.15 and 0.15, with StRefHISH4, StRefActin 1, αTub, and EF1αbeing the best trustworthy expressed genes. In distinct organs and fruit development stages subject to salt stress, the most stable genes were determined as StRefHISH4 and StRefActin 1 according to M values in all samples, and the M value was found to be below nearly 0.15 in all samples.

As seen in Figure 5, it appears that including a third housekeeping gene in this study did not play an important part in the changing of the standardization factor (V2/V3 < 0.15) in all organs and circumstances tested, and the two-reference gene has been seen enough for normalization. To work with the correct number of reference genes, geNorm analysis was applied to appraise the number of housekeeping genes utilized in standardization under diverse circumstances. Pairwise variation (Vn/Vn+1) across the consecutive sequenced standardization factors (NFn and NFn+1, n≥2) was evaluated by geNorm analysis (Han et al., 2012). The limit rate is 0.15, under which including an extra check gene is not essential for credible standardization (Cassan-Wang et al., 2012b). As shown in Figure 6, from the results of the geNorm analysis, the least steady gene and the steadiest gene were determined. It is necessary to use more than one housekeeping gene, as one reference gene cannot properly standardize gene expression in different organs of plants, in different growth conditions, or even in different cultivars (Vandesompele et al., 2002; Gimenez et al., 2011).

3.2.3. NormFinder analysis

Standardization factor in NormFinder was evaluated within and between groups (Andersen et al., 2004). The classification of the genes and their separate expression

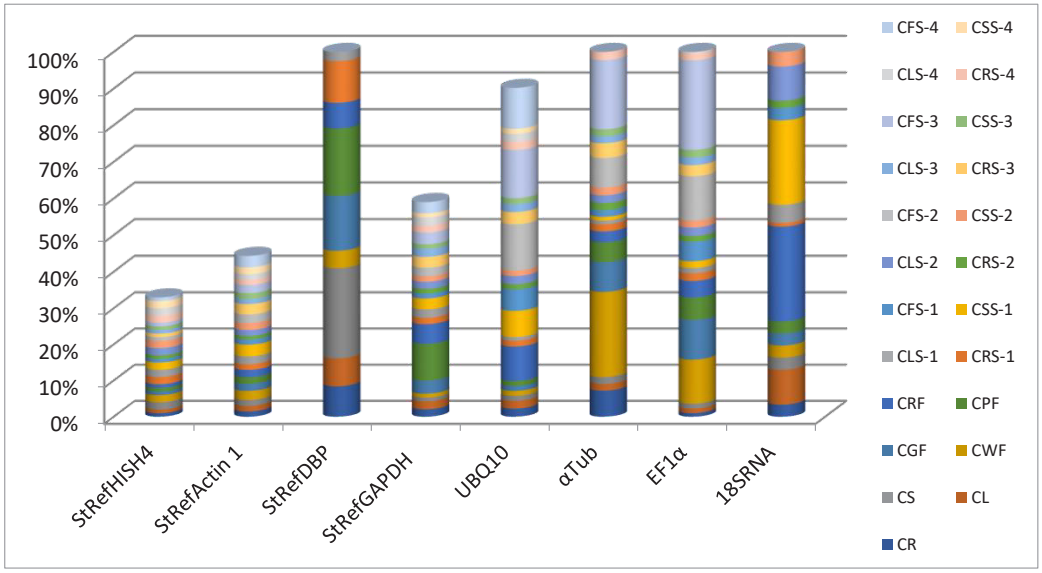


Figure 3. Relative expression differences of 8 nominee housekeeping genes in various samples of the ''Camarosa'' cultivar. CR: ''Camarosa'' root; CL: ''Camarosa'' leaf; CS: ''Camarosa'' stem; CWF: ''Camarosa'' white fruit; CGF: ''Camarosa'' green fruit; CPF: ''Camarosa'' pink fruit; CRF: ''Camarosa'' red fruit; CRS-1: ''Camarosa'' root 1st salt application; CLS-1: ''Camarosa'' leaf 1st salt application; CSS-1: ''Camarosa'' stem 1st salt application; CFS-1: ''Camarosa'' fruit 1st salt application; CRS-2: ''Camarosa'' root 2nd salt application; CLS-2: ''Camarosa'' leaf 2nd salt application; CSS-2: ''Camarosa'' stem 2nd salt application; CFS-2: ''Camarosa'' fruit 2nd salt application; CRS-3: ''Camarosa'' root 3rd salt application; CLS-3: ''Camarosa'' leaf 3nd salt application; CSS-3: ''Camarosa'' stem 3st salt application; CFS-3: ''Camarosa'' fruit 3rd salt application; CRS-4: ''Camarosa'' root 4th salt application; CLS-4: ''Camarosa'' leaf 4th salt application; CSS-4: ''Camarosa'' stem 4th salt application; CFS-4: ''Camarosa'' fruit 4th salt application.

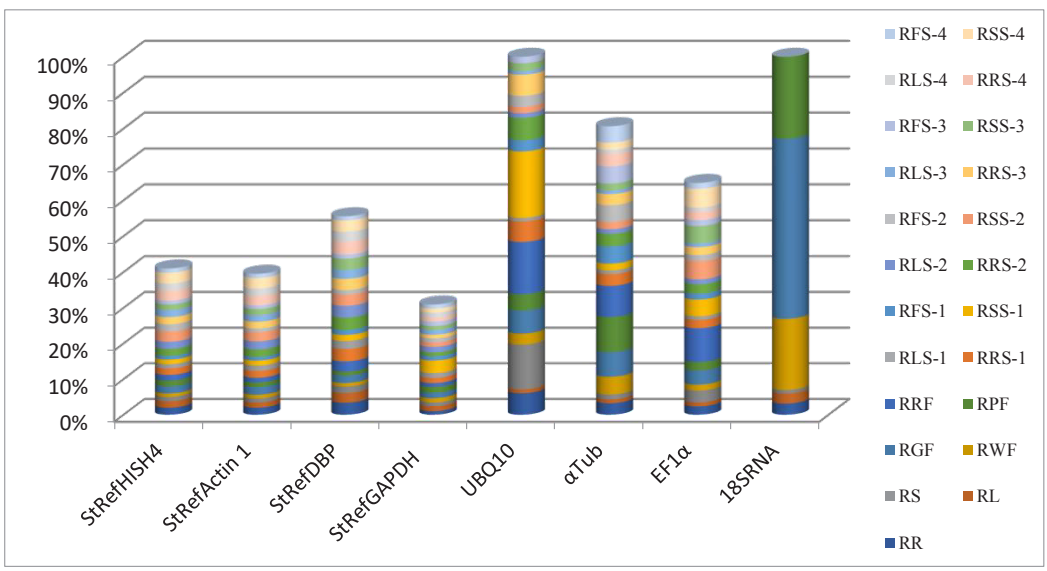
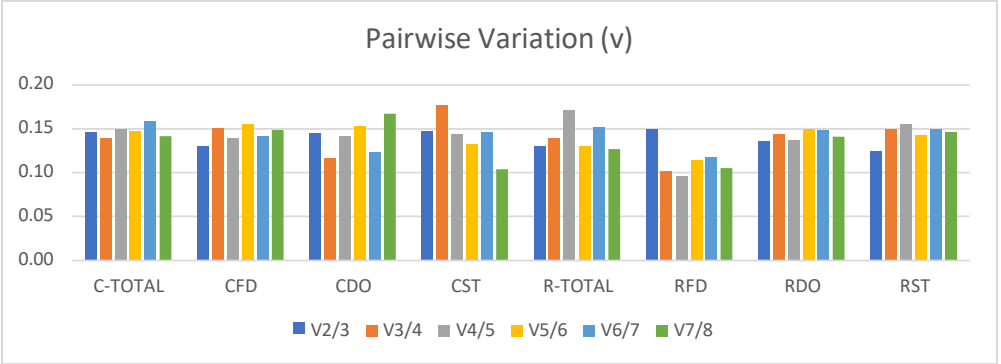


Figure 4. Relative expression differences of 8 nominee housekeeping genes in various samples of the ''Rubygem'' cultivar. RR: ''Rubygem'' root; RL: ''Rubygem'' leaf; RS: ''Rubygem'' stem; RWF: ''Rubygem'' white fruit; RGF: ''Rubygem'' green fruit; RPF: ''Rubygem'' pink fruit; RRF: ''Rubygem'' red fruit; RRS-1: ''Rubygem'' root fruit 1st salt application; RLS-1: ''Rubygem'' leaf fruit 1st salt application; RSS-1: ''Rubygem'' stem 1st salt application; RFS-1: ''Rubygem'' fruit 1st salt application; RRS-2: ''Rubygem'' root 2nd salt application; RLS-2: ''Rubygem'' leaf 2nd salt application; RSS-2: ''Rubygem'' stem 2nd salt application; RFS-1: ''Rubygem'' fruit 1st salt application; RRS-3: ''Rubygem'' root 3rd salt application; RLS-3: ''Rubygem'' leaf 3rd salt application; RSS-3: ''Rubygem'' stem 3rd salt application; RFS-3: ''Rubygem'' fruit 3rd salt application; RRS-4: ''Rubygem'' root 4th salt application; RLS-4: ''Rubygem'' leaf 4th salt application; RSS-4: ''Rubygem'' stem 4th salt application; RFS-4: ''Rubygem'' fruit 4th salt application.



	C-TOTAL	CFD	CDO	CST	R-TOTAL	RFD	RDO	RST
V2/3	0.147	0.13	0.145	0.148	0.13	0.15	0.136	0.125
V3/4	0.14	0.151	0.117	0.177	0.139	0.102	0.144	0.15
V4/5	0.15	0.14	0.142	0.144	0.171	0.096	0.138	0.155
V5/6	0.148	0.156	0.153	0.133	0.131	0.114	0.15	0.143
V6/7	0.159	0.142	0.124	0.146	0.152	0.118	0.149	0.15
V7/8	0.142	0.149	0.167	0.104	0.127	0.105	0.141	0.147

Figure 5. A binary variation (Vn/Vn+1) study across normalization factors (NFn and NFn+1) was applied with the geNorm program to define the right count of reference genes that can be chosen for RT-qPCR data standardization in two different cultivars, different organs, fruits at different growth stages, and salt-treated samples at different growth stages. C-Total: all plant samples in ''Camarosa''; CFD: ''Camarosa'' different fruit development stages; CDO: ''Camarosa'' different organs; CST: ''Camarosa'' salt treatments. R-Total: all plant samples in ''Rubygem''; RFD: ''Rubygem'' different fruit development stages; RDO: ''Rubygem'' different organs; RST: ''Rubygem'' salt treatments.

stability values (SV) are seen for ''Camarosa'' in Table 2 and ''Rubygem'' in Table 3. In accordance with NormFinder, the steady values of two reference genes in both cultivars were most steadily expressed in different organs and different fruit development periods, plant tissues exposed to salinity, and in all samples. Genes StRefActin 1 was most stably expressed in different organs (SV = 0.289) and at different fruit development stages (SV = 1.601) in ''Camarosa'', while StRefHIS4 in plant tissues exposed to salinity (SV = 0.362) and in all samples StRefHIS4 (SV = 0.295) were the most stably expressed genes. The least stable genes were determined as StRefDBP (SV = 6.504) in different organs, 18SRNA at different fruit development stages (SV = 12.190), plant tissues exposed to salinity (SV = 15.145), and in all samples (SV = 14.068).

According to NormFinder, the most steadily expressed gene was StRefHISH4 in all analyzed samples in ''Rubygem''. SV were found for different organs SV = 0.273, different fruit development periods SV = 0.247, plant tissues exposed to salinity SV = 0.125, and all samples SV = 0.225. UBQ10 (SV = 6.158) was the least steady gene in different organs, while 18SRNA was the least steady gene in varied fruit development phases (SV = 14.252), plant tissues exposed to salinity (SV = 12.698) and all samples (SV = 1.659).

3.2.4. ΔCt method and Bestkeeper analysis

DCt method was utilized to order the nominee housekeeping genes relying on mean standard deviation (SD). This method calculates by comparing the relative expression of gene pairs in every pattern (Silver et al., 2006). As displayed in Tables 2 and 3, StRefHISH4 and StRefACT 1 genes are the steadiest reference genes in 2 different cultivars and different experimental groups. According to DCt method, while the least stable genes were αTub, UBQ10, and StRefDBP genes in ''Camarosa'', the least stable genes were determined as 18SRNA, StRefDBP, and StRefGAPDH in ''Rubigem''.

BestKeeper analysis was utilized to identify the steadiness of gene expression relying on SD and coefficient of variation (CV) rates attained using Cq values of housekeeping genes (Pfaffl et al., 2004). The SD ratio is oppositely proportional to gene steadiness meaning that genes are more specific and have lower SD rates. Accordingly, StRefHIS4 (SD = 0.24 and SD = 0.00), both in different organs and different fruit development periods, were determined as the most steadily expressed genes, respectively. In ''Camarosa'', StRefACT 1 (SD = 0.33 and SD = 0.32) gene was defined as the most specific gene in plant tissues exposed to salinity and in all samples, respectively. The least stable genes were EF1α, UBQ10 and

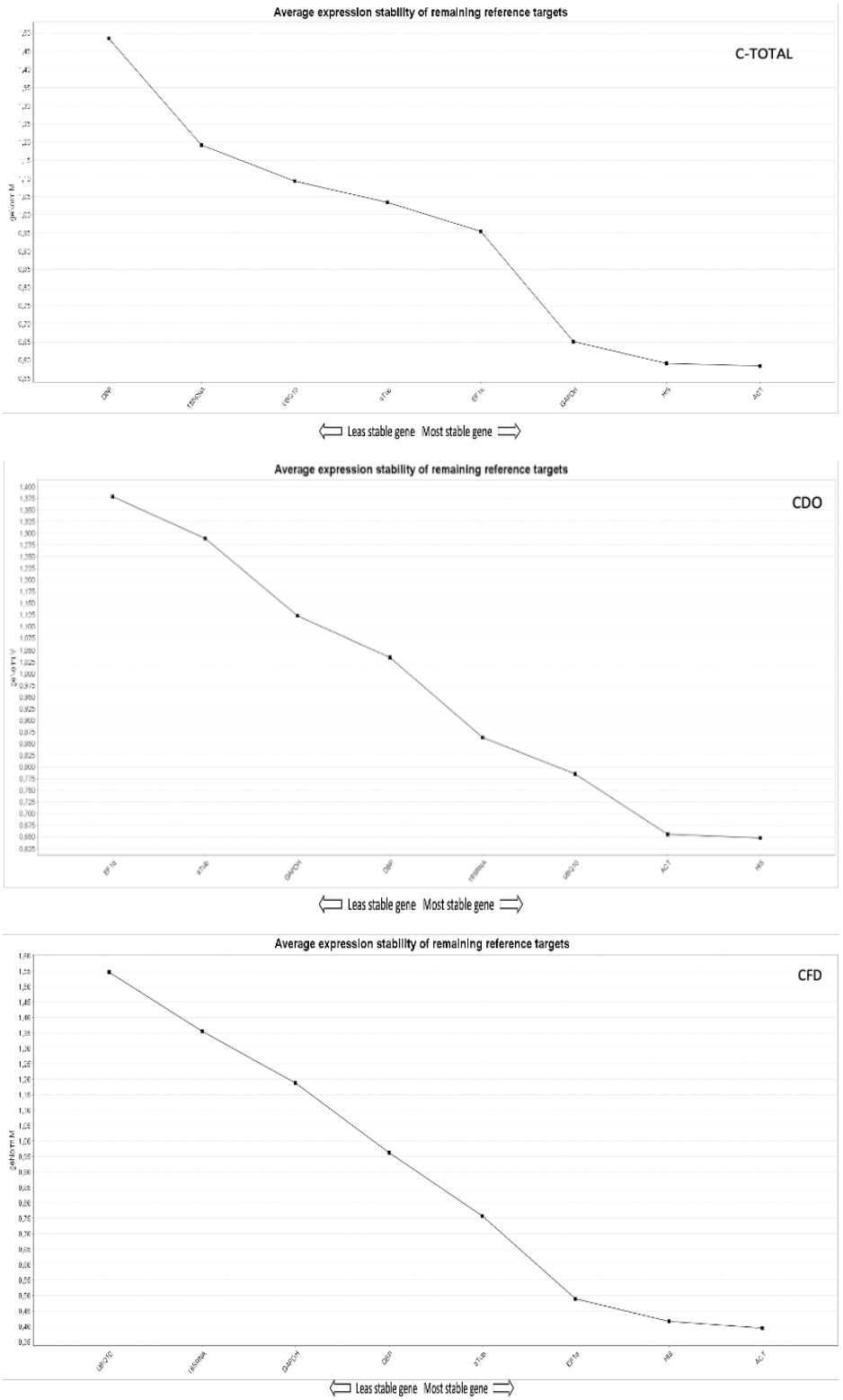


Figure 6. Average expression stability (M value) of 8 genes assessed with geNorm analysis. Expression steadiness was assessed in ''Camarosa'', all plant samples in ''Camarosa'' (C-total), different organs (CDO), ''Camarosa'' different fruit development periods (CFD), and ''Camarosa' salt treatments (CST) samples. The lower M rate is, the more steady expression of the reference gene. All plant samples in ''Rubygem'' (R-Total), ''Rubygem'' different organs (RDO), ''Rubygem'' different fruit development stages (RFD), and ''Rubygem'' salt treatments (RST).

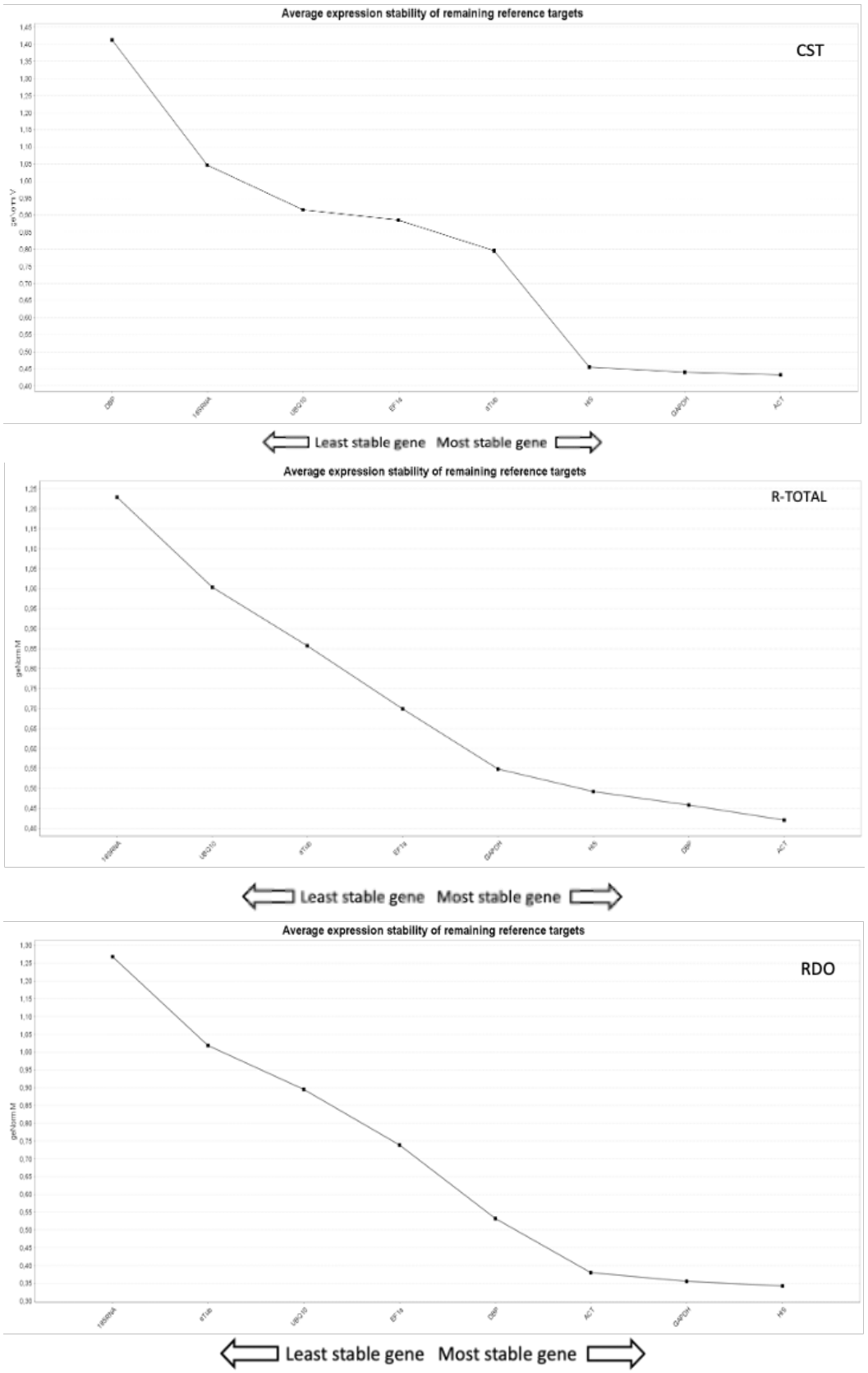


Figure 6. (Continued).

Table 2. Gene stability and standard deviation obtained by using 4 package programs of different organs, different fruit development periods, and different tissues in different development periods treated with salt in ''Camarosa''.

		BestKeeper		**GeNorm**		**Delta CT**		**NormFinder**	
		Gene	SD dev [± CP]	Gene	Stability	Gene	Stability	Gene	Stability
Different organs	1	StRefHISH4	0.24	StRefHISH4	0.010	StRefActin 1	1.06	StRefActin1	0.289
	2	StRefActin1	0.29	UBQ10	0.101	StRefHISH4	1.15	StRefHISH4	0.289
	3	StRefDBP	0.78	StRefGAPDH	0.101	18SRNA	1.33	StRefGAPDH	0.289
	4	18SRNA	0.82	EF1α	0.385	StRefGAPDH	1.41	UBQ10	0.289
	5	UBQ10	0.98	StRefActin1	0.622	StRefDBP	1.50	EF1α	0.289
	6	αTub	1.06	αTub	1.537	UBQ10	1.53	αTub	3.653
	7	StRefGAPDH	1.06	18SRNA	2.453	EF1α	1.63	18SRNA	4.713
	8	EF1α	1.39	StRefDBP	4.801	αTub	1.64	StRefDBP	6.504
Different fruit development periods	1	StRefHISH4	0.00	StRefHISH4	0.10	StRefActin 1	1.07	StRefActin1	1.601
	2	StRefActin1	0.38	StRefActin1	3.202	StRefHISH4	1.14	StRefHISH4	1.161
	3	EF1α	0.50	EF1α	3.202	StRefDBP	1.32	UBQ10	2.932
	4	StRefDBP	0.75	StRefGAPDH	4.691	EF1α	1.38	StRefGAPDH	3.556
	5	αTub	0.75	UBQ10	5.323	18SRNA	1.44	EF1α	3.584
	6	StRefGAPDH	1.00	StRefDBP	5.956	StRefGAPDH	1.55	StRefDBP	7.532
	7	18SRNA	1.13	αTub	7.643	αTub	1.68	αTub	11.105
	8	UBQ10	1.50	18SRNA	9.193	UBQ10	1.82	18SRNA	12.190
Plant tissues exposed to salinity	1	StRefActin1	0.33	StRefHISH4	0.574	StRefActin 1	1.13	StRefHISH4	0.362
	2	StRefGAPDH	0.38	StRefActin1	0.574	StRefGAPDH	1.14	αTub	0.880
	3	StRefHISH4	0.43	StRefGAPDH	0.574	StRefHISH4	1.24	UBQ10	1.455
	4	αTub	0.88	UBQ10	2.927	αTub	1.24	EF1α	1.649
	5	18SRNA	0.95	αTub	3.577	UBQ10	1.25	StRefGAPDH	3.603
	6	UBQ10	1.05	EF1α	3.920	EF1α	1.32	StRefActin1	3.837
	7	EF1α	1.09	StRefDBP	5.858	18SRNA	1.59	StRefDBP	10.608
	8	StRefDBP	1.97	18SRNA	8.630	StRefDBP	2.40	18SRNA	15.145
All samples	1	StRefActin1	0.32	StRefHISH4	0.582	StRefActin 1	1.15	StRefHISH4	0.295
	2	StRefHISH4	0.51	StRefActin1	0.582	StRefHISH4	1.24	UBQ10	1.987
	3	StRefGAPDH	0.55	StRefGAPDH	1.928	StRefGAPDH	1.26	EF1α	2.165
	4	18SRNA	0.96	UBQ10	3.397	αTub	1.44	StRefActin1	2.860
	5	UBQ10	1.02	EF1α	4.354	UBQ10	1.44	StRefGAPDH	3.201
	6	αTub	1.07	αTub	4.742	EF1α	1.45	αTub	4.451
	7	EF1α	1.21	StRefDBP	6.383	18SRNA	1.59	StRefDBP	10.234
	8	StRefDBP	1.80	18SRNA	8.748	StRefDBP	2.25	18SRNA	14.068

StRefDBP in ''Camarosa''. In ''Rubigem'', on the other hand, the most specific gene for StRefACT 1 was found both in different organs (SD = 0.29), and plant tissues exposed to salinity (SD = 0.41) and different fruit development stages (SD = 0.28). 18SRNA (SD = 1.97), EF1α (SD = 0.75), and StRefGAPDH (SD = 4.02) were the least stable genes in ''Rubygem'' (Table 3). As a result, RefFinder was performed to identify suitable housekeeping genes (Xie et al., 2012). According to the RefFinder results, the StRefHIS4 and StRefACT 1 genes were the steadiest expressed, when all the samples were taken into account. These results are coherent with all analyses performed.

Reference genes have been formerly defined in strawberries (Galli et al., 2015; Amil-Ruiz et al., 2013;

Table 3. Gene stability and standard deviation obtained by using 4 package programs of different organs, different fruit development periods, and different tissues in different development periods treated with salt in ''Rubygem''.

		BestKeeper		**GeNorm**		**Delta CT**		**NormFinder**	
		Gene	SD dev [± CP]	Gene	Stability	Gene	Stability	Gene	Stability
Different organs	1	StRefActin1	0.29	StRefHISH4	0.569	StRefHISH4	1.21	StRefHISH4	0.273
	2	StRefGAPDH	0.44	StRefActin1	0.577	StRefGAPDH	1.24	StRefGAPDH	0.289
	3	StRefHISH4	0.47	StRefDBP	0.577	StRefActin1	1.25	StRefActin 1	0.289
	4	StRefDBP	0.65	StRefGAPDH	0.577	EF1α	1.41	EF1α	0.500
	5	EF1α	1.03	18SRNA	0.744	αTub	1.45	αTub	0.850
	6	UBQ10	1.09	αTub	0.835	StRefDBP	1.56	StRefDBP	1.059
	7	αTub	1.27	EF1α	1.043	UBQ10	1.66	18SRNA	1.841
	8	18SRNA	1.97	UBQ10	2.510	18SRNA	2.17	UBQ10	6.158
Different fruit development periods	1	StRefActin1	0.28	StRefHISH4	0.357	StRefHISH4	0.81	StRefHISH4	0.247
	2	StRefGAPDH	0.28	StRefActin1	0.500	StRefActin1	0.84	StRefActin 1	0.250
	3	StRefHISH4	0.44	StRefGAPDH	0.500	αTub	0.85	StRefGAPDH	0.401
	4	αTub	0.49	StRefDBP	0.919	StRefGAPDH	0.90	StRefDBP	0.500
	5	StRefDBP	0.64	αTub	1.560	UBQ10	1.04	αTub	1.978
	6	18SRNA	0.67	EF1α	2.202	18SRNA	1.09	EF1α	2.431
	7	UBQ10	0.72	UBQ10	2.950	EF1α	1.10	UBQ10	5.011
	8	EF1α	0.75	18SRNA	6.227	StRefDBP	1.13	18SRNA	14.252
Plant tissues exposed to salinity	1	StRefActin1	0.38	StRefHISH4	0.250	StRefHISH4	2.00	StRefHISH4	0.125
	2	StRefHISH4	0.41	StRefActin1	0.647	StRefActin1	2.11	StRefGAPDH	0.618
	3	StRefDBP	0.50	StRefDBP	0.647	EF1α	2.11	EF1α	0.964
	4	EF1α	0.75	StRefGAPDH	0.989	StRefDBP	2.12	αTub	1.296
	5	αTub	1.00	EF1α	1.485	αTub	2.13	StRefActin 1	1.500
	6	UBQ10	1.31	αTub	2.017	18SRNA	2.73	StRefDBP	2.013
	7	18SRNA	1.38	UBQ10	3.672	UBQ10	2.76	UBQ10	7.517
	8	StRefGAPDH	4.02	18SRNA	6.331	StRefGAPDH	8.56	18SRNA	12.698
All samples	1	StRefActin1	0.41	StRefActin1	0.426	StRefHISH4	0.92	StRefHISH4	0.225
	2	StRefDBP	0.41	StRefDBP	0.426	GAPDH	1.00	StRefGAPDH	0.411
	3	StRefHISH4	0.48	StRefHISH4	0.503	EF1α	1.04	EF1α	0.498
	4	StRefGAPDH	0.49	StRefGAPDH	0.598	StRefActin1	1.10	αTub	0.685
	5	EF1α	0.75	EF1α	0.720	StRefDBP	1.10	StRefActin 1	0.767
	6	αTub	1.12	αTub	0.849	αTub	1.16	StRefDBP	0.786
	7	UBQ10	1.19	UBQ10	1.020	UBQ10	1.59	UBQ10	1.417
	8	18SRNA	1.66	18SRNA	1.213	18SRNA	1.79	18SRNA	1.659

Zhang et al., 2018; Liu et al., 2019; Chen et al., 2021; Ye et al., 2021). However, it has been claimed that the previously determined reference genes vary according to strawberry cultivars, the organ used, and different stress conditions. However, when the results obtained in this study were examined, no change was observed in the steadiest housekeeping genes by evaluating the results of RT-qPCR performed in organs at different developmental stages, fruits at varied developmental phases, under salt stress applied, and all samples.

3.2.4 Validating the expression levels of candidate reference genes by RT-qPCR

With RT-qPCR, the specificity of the primers utilized for housekeeping genes was confirmed. A band for each

gene was shown with electrophoresis, in the absence of primer-dimers or nonspecific amplification. Relying on SYBR Green find, RT-qPCR studies were used to assess the expression steadiness of the eight housekeeping genes in different organs, different developmental stages, and plant tissues of two strawberry cultivars treated with salinity. The samples were separated into four groups including four organs (roots, stem, leaves, and fruits), four developmental periods, and in different organs under salt stress. Then the Ct values of the housekeeping genes of each group were utilized to determine the various degrees of expression. Expression coefficients of reference genes were calculated with 2-deltaCt, and expressions of primers were determined. It was also validated in the most stable genes with statistical analysis results.

4. Discussion

Appropriate reference gene selection indicates a stable expression rate in the studied samples (Liu et al., 2014). RT-qPCR has been extensively used for gene expression studies in high-throughput transcriptomic (Wong and Medrano, 2005). A secure reference gene should indicate the least variation in studies. Defining the stability of expression of housekeeping genes beforehand and standardizing the expression coefficients of the genes to be studied is very important for the correct explanation of RT-qPCR results (Hamalainen et al., 2001). Our Cq rates were used to define the expression levels of conventional housekeeping genes and new candidate housekeeping genes. In the study, we evaluated two different strawberry cultivars, ''Camarosa" and ''Rubygem", using different organs, fruits at different developmental stages, different organs exposed to salt, and all the samples. The results determined that StRefACT 1 and StRefHISH4 had minimum Cq values and the most highly expressed genes in two different strawberry cultivars (20.73 and 19.30, respectively, in ''Camarosa" and 20.47 and 19.14, respectively, in ''Rubygem"). Moreover, in ''Camarosa" and ''Rubygem", 18SRNA was the least expressed gene in samples of the two cultivars, with the highest mean Cq value (42.98 and 42.01, respectively).

Reference gene studies have been carried out on different plant species before (Le et al., 2012; Liu et al., 2014; 2015; 2022; Whang et al., 2017; Almas-Kamrodi et al., 2018; Umadevi et al., 2019; Dong et al., 2022; Li et al., 2022; Zong et al., 2022). Reference gene studies are also available in strawberry. Galli et al.'s (2015) candidate reference gene evaluation study was carried out in different strawberry cultivars, but the steadiest housekeeping genes were determined as different genes in different statistical evaluations. As a result of our study, the same reference genes (StRefHISH4 and StRefACT1) were consistently determined as the most stable in 2 different cultivars. Zhang et al. (2018) conducted a study similar to ours. Seven reference genes were identified with different tissues, different fruit development stages, light quality, and low temperature applications. However, unlike our work, they stated that the data they obtained was not stable under different experimental conditions of the genes used. A similar result was reported by Liu et al. (2019). They stated that the steadiness of housekeeping genes changed depending on diverse stress circumstances and developmental stages of strawberry. It was determined that the commonly used reference genes we used in our study were less stable in strawberry than the reference genes obtained from the sequences of *F. vesca* and *F. x ananassa*. Chen et al. (2021) determined in their study that commonly used reference genes are not stable for normalization during fruit development in strawberry cultivars. They compared 9 new candidate reference genes obtained from existing RNAseq data in receptacle development with commonly used genes. As consistent with our work, they determined that the novel candidate housekeeping genes are steadier than the commonly used reference genes. In Ye et al.'s study (2021), strawberry (*Fragaria x ananassa*) seedlings were exposed to diverse stress circumstances. Expression levels of seven housekeeping genes in strawberry leaves were examined by RT-qPCR. Expression steadiness of candidate housekeeping genes was measured with geNorm, NormFinder, BestKeeper, and RefFinder programs. Contrary to our results, they claimed that the expression steadiness of housekeeping genes altered under varied circunstances. FaACTIN2 was valued as the best steady reference gene for cold and white light stress. FaGAPDH was determined as the housekeeping gene in plants treated with salt stress conditions and red light. Under drought stress, FaDBP was determined as the housekeeping gene with maximum expression stability. It was determined that there was a stable reference gene for FaHISTH4 in plants treated with heat stress and blue light. Contrary to their study, our outcomes indicated that the same reference genes were the most stable in different organs, fruits at varied developmental phases, and salt-stressed strawberry plants in both strawberry cultivars. Moreover, StRefHISH4 and StRefACT 1 genes were found to be the most stable genes according to the results of RT-qPCR performed on different organs and exposed to salt during the fruit ripening period of two different cultivars. The least stable genes were found as StRefDBP and 18SRNA in plant tissues exposed to salinity in ''Camarosa", while StRefGAPDH and 18SRNA were identified as the least steady genes in ''Rubygem".

In this study, by using the validation of RefFinder analysis, important results were obtained for gene expression analyses in future breeding studies and RNAseq studies in terms of choosing the correct reference genes in future studies on strawberries.

5. Conclusions

Gene expression investigation can supply basic proof of gene activations in reply to exterior circumstance stress. Furthermore, quantitative RT-qPCR has been broadly

utilized in gene expression research owing to its speed, sensitivity, correctness, and quantification. Therefore, the most appropriate and most accurate reference gene should be selected in gene expression studies in order for the results to be accurate and reliable. Assessing and choosing a suitable reference gene lays the foundation for accurately researching RT-qPCR data. In this research, we assessed eight reference genes based on their expression steadiness in root, stem, leaf, and fruits organs and being exposed to salt stress circumstances. 18SRNA was not appropriate as a reference gene because of its weak steadiness and extreme expression level in all samples. For distinct outer circumstances, a particular housekeeping gene utilization should rely on its expression steadiness under a certain situation. StRefACTI 1 and StRefHISH4 were identified to be the steadiest housekeeping gene for all samples. Our results provided a basis for examining different organs and salt stress-related gene functions in strawberries by identifying reference genes that can be used in future transcriptome studies.

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