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Evaluation And Validation of Reliable Reference Genes For Quantitative Real-Time PCR Analysis of The Gene Expression In *Macadamia Integrifolia*

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Abstract

Background: *Macadamia integrifolia*, a new economically important crop, the kernel oil is rich in bioactive compound and monounsaturated fatty acid. Gene expression analysis of qRT-PCR is beneficial to understand the complex regulatory networks of macadamia.

Results: In this study, the expression stability of 11 traditional housekeeping genes including α-tubulin (*TUBa*), β–tubulin (*TUBb*), malate dehydrogenase (*MDH*), 18S ribosome RNA (*18S*), *glyceraldehyde-3- phosphate dehydrogenase* (*GAPDH*), *α-elongation factor 1* (*EF1a*), *β- elongation factor 1* (*EF1b*), *ubiquitin* (*UBQ*), *ubiquitin-conjugating enzyme* (*UBC*), *cyclophilin* (*CYP*) and *actin* (*ACT*) were accessed by qRT-PCR in macadamia seedlings under different experimental conditions and tissues. The expression stability of the 11 reference genes was evaluated by the online tool RefFinder, which include ΔCt, geNorm, NormFinder, BestKeeper four commonly software, and then determinated a comprehensive expression stability ranking by integrating above four ranking results based on the geometric mean. Our results show that *ACT* was the best stable genes for all samples, cold stress, NaCl sress, PEG stress, ABA treatment, MeJA treatment, stem and leaf tissue samples; *EF1b* is the most stable gene in GA treatment and heat stress samples; *UBC* and *CYP* were respectively ranked top in ethylene treatment and root tissue samples. Finally, the reliability of these results was further validated with a target gene SAD by qRT-PCR.

Conclusions: In summary, this study evaluated and validated the suitable reference genes for qRT-PCR under different experiment treatment and tissues, and will be useful for further gene expression studies on the molecular mechanisms in Macadamia.

Background

Macadamia is a genus that belongs to the Proteaceae family, is a long-lived evergreen tree of subtropical and tropical origin, and native to Australia. The commercial cultivars is composed of *Macadamia integrifolia Maiden and Bethche*, *Macadamia tetraphylla* and their hybrids[1]. Macadamia produce edible dried fruit[2], and also are consumed as an ingredient in making pastry, chocolate, oil, cosmetic and pharmaceutical industry. Therefore macadamia is the most economically important crop. Macadamias have also been expanded in South Africa, Kenya, China, the United State, Guatemala, Malawi, Brazil, Vietnam, Mexico and Thailand currently. In China the planting area reach to 295856 hm^2 until 2018, and account for more than 65% of the total area of the world grow, becoming the world's largest country with planting area.

The shells of *Macadamia* nut is very hard to crack as the high strength and toughness, it is considered as an interesting research material to study for developing of impact- and puncture-resistant engineering material[3]. Moreover, tocotrienols[4] and squalene[5] these bioactive compounds were detected in macadamia oil, and these compounds with high antioxidant ability can be beneficial to health maintenance and disease prevention[6]. Diets containing macadamia nuts reduce plasma LDL cholesterol levels and decrease the risk of CVD[7]. The kernel of macadamia nut is rich of lipids. Macadamia oil contains higher monounsaturated fatty acid than other vegetable oil[5, 8], and hence oil content as a major indicator of macadamia kernel quality, but the mechanisms accumulating the unsaturated fatty acid are still unknown. Gene expression analysis of fatty acid synthesis is beneficial to understand the complex regulatory networks of macadamia oil accumulation. In macadamia, soluble acy-ACP desaturases[9–10] and thiesterases[11]which were responsible for accumulation of unsaturated fatty acids were cloned and characterized, but the transcriptional level regulation need to be further elucidate. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is one of the most frequently-used technologies to study the expression pattern of genes[12–13]. And it was widely used in the analysis of gene expression levels with high sensitivity, specificity, reproducibility and accuracy[14]. The accuracy of qRT-PCR results depends largely on the selection of internal reference genes. Selecting suitable reference genes as internal control under different experimental conditions is very necessary because the starting amount of material, RNA integrity, reverse transcription efficiency, cDNA quality, and different tissue and cell type can significantly influence the accuracy of gene expression [15–16]. Studies have shown that the expression of housekeeping genes is not constant in different cell types and physiological states. The expression of housekeeping genes will change with the change of experimental conditions and organ specificity. If the change of internal reference gene expression is not considered, the accuracy of qRT-PCR results will be reduced or even the opposite or wrong conclusion will be reached. Therefore, in order to decipher the molecular mechanisms of UFAs biosynthesis (accumulation) promoted by *SAD* and *FAT* genes in *Macadamia integrifolia* kernel, the need for selecting suitable reference gene for qRT-PCR in macadamia is urgent .

In this study, we selected 11 traditional candidate reference genes, α-tubulin (*TUBa*), β–tubulin (*TUBb*), malate dehydrogenase (*MDH*), 18S ribosome RNA (*18S*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *α-elongation factor 1* (*EF1a*), *β- elongation factor 1*

(*EF1b*), *ubiquitin* (*UBQ*), *ubiquitin-conjugating enzyme* (*UBC*), *cyclophilin* (*CYP*) and *actin* (*ACT*), tested its expression pattern under various treatments by qRT-PCR. The expression stability of these candidate reference genes were further validated and evaluated using statistical algorithms includingΔCt[17], geNrom[18], Normfinder[19], and BestKeeper[20]. The comprehensive stability ranking of these reference genes under each specific experimental condition were also performed. Additionally, one target gene, *Δ9-Stearoyl-ACP desaturases* (*SAD*) which first desaturation introduction in the synthesis of aliphatic chains in plant was used to validate the effectiveness of the selected reference genes. Finally, this work provides the basis for further research in exploring of gene expression profiling in macadamia oil accumulation.

Results

1. Verification of primer specificity and PCR efficiency

In the present study, 11 traditional housekeeping genes were chosen as candidate reference genes used in qRT-PCR studies namely, *18S ribosome RNA*(*18S rRNA*), *Actin*(*ACT*), *Cyclophilin*(*CYP*), *Elongation factor 1-α(EF1a*), *Elongation factor 1-β*(*EF1b*), *Glyceraldehyde-3-phosphate dehydrogenase*(*GAPDH*), *Malate dehydrogenease* (*MDH*), *Ubiquitin-conjugating enzyme*(*UBC*), *Tubulin-α*(*TUBa*), *Tubulin -β*(*TUBb*), and *Ubiquitin-A*(*UBQ*) in various conditions(**Supplemental Table 1**). The primers sequences of 11 candidate reference were describe in Table 1. The melting curve analysis following qRT-PCR presented a single peak, indicated that the specificity of primers (**Supplementary Fig. 1**). The amplification efficiency of primers were assessed by qRT-PCR after 45 cycles from all sample under different gradient concentrations (**Supplementary Fig. 2**), ranged from 91.9-102.5%, and the correlation coefficient (R^2) near 0.99 (Table 1**)**.

Table 1
Primers sequences of the candidate reference genes

Gene name	Genes symbol	Gene ID	Primers sequences(5'-3')	Primer Efficiency	R^2
18S ribosome RNA	*18S*	MN650750	F: CTGAGAAACGGCTACCACATC	102.51	0.963
			R: CGAAGAGCCCGGTATTGTTATT		
ACT-β	*ACT*	MN627205	F: GAGGAGAGGATCTGTCGTAAA	91.96	0.998
			R: GATAACAAGGAGAGGCCAAAG		
Cyclophilin	*CYP*	MN627206	F: AACAAGTTCGCCGATGAG	95.05	0.999
			R: GTCTTCGCAGTGCAAATAAAG		
Elongation factor 1-α	*EF1a*	MN627207	F: CCCACTTCAGGGTGTTTAC	97.99	0.994
			R: CGAAGGTGACAACCATACC		
Elongation factor 1-β	*EF1b*	MN627208	F: GGCTGCTAAAGCATCTACAA	94.41	0.998
			R: CGAACAGCTTCCTCTAGTTTC		
Glyceraldehyde 3-phosphate dehydrogenase	*GADPH*	MN627209	F: GTTGGTGACTGTAGGTCAAG	93.73	0.999
			R: AGGTCCAACACTCGGTTA		
Malate dehydrogenease	*MADH*	MN627210	F: GCTGGTCTCATCTATTCTTTCC	98.61	0.999
			R: CGTCCAACTTCTTCCTTGAG		
Tubulin-α	*TUBa*	MN627211	F: GGCTTGTGTCTCAGGTTATT	94.9	0.999
			R: GTGGATATGGGACCAAGTTAG		
Tubulin-β	*TUBb*	MN627212	F: ATATGAGGATGAGGAGGAAGG	98.35	0.999
			R: CCCATAATCAGCCACTGTAAA		
Ubiquitin	*UBQ*	MN627213	F: GTGGATGTTGATGGATGAAAC	98.68	0.999
			R: GTACTTACAGAGCGTCCTTAC		
Ubiquitin-conjugating enzyme	*UBC*	MN627214	F: CCACCCAAGGTAGCATTTAG	95.95	0.982
			R: CTGGGCTCCATTGTTCTTTA		

2. Expression profile of the candidate reference genes

Cycle threshold (Ct) value of qRT-PCR is the number of cycles when the fluorescence signal intensity reach a set threshold level of detection, and it represents the transcript levels of genes in test samples. The transcript level of 11 reference genes exhibited a relatively high variation from all the experimental set as show in **Supplemental Table 2**. The median values of Ct ranged from 16.73 for *18S rRNA* to 26.41 for *GAPDH,* most values lying between 20 and 23 across all samples. *18S rRNA* showed the most high expression level with the lowest mean Ct value (17.66±2.62)followed by *CYP*(21.05±2.39), *MDH*(21.97±2.26), *ACT*(22.82±2.20), *UBQ*(23.05±3.08), *UBC*(23.25±2.39), *EF1a*(23.79±2.98), *EF1b*(23.80±2.41), *TUBb*(25.40±2.21), *GAPDH*(27.69±2.86). The expression of *TUBa*(27.94±3.22) candidate gene was found to be the lowest level from all the test sample. The genes with higher SD of Ct values

indicated more variable expression compared to these with lower SD. *ACT* showed the smallest variation in gene expression with lowest SD(22.82±2.20), while *TUBa*(27.94±3.22) appearance the most variable levels of expression(**Figure 1**).

3. Expression stability analysis of candidate reference genes

In order to select the best reference genes, RefFinder online tool was used to rank the expression stability values. This method include comparative ΔCt, geNorm, NormFinder and BestKeeper, four popular algorithms, determinates a comprehensive expression stability ranking combined with above four data generated from different algorithms(**Fig. 2; Table 2**).

Table 2
Expression stability ranking of the 11 candidate reference genes.

Method/Rank	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
(A)Ranking order under cold stress(better-good-average)											
Delta CT	*ACT*	*GAPDH*	*CYP*	*EF1b*	*MDH*	*TUBb*	*UBC*	*EF1a*	*18S*	*UBQ*	*TUBa*
BestKeeper	*UBC*	*TUBb*	*EF1b*	*ACT*	*CYP*	*MDH*	*GAPDH*	*TUBa*	*EF1a*	*UBQ*	*18S*
NormFinder	*ACT*	*GAPDH*	*CYP*	*EF1b*	*TUBb*	*MDH*	*UBC*	*EF1a*	*18S*	*TUBa*	*UBQ*
geNorm	*ACT / EF1b*		*GAPDH*	*CYP*	*MDH*	*TUBb*	*UBC*	*18S*	*EF1a*	*UBQ*	*TUBa*
Comprehensive Ranking	*ACT*	*EF1b*	*GAPDH*	*CYP*	*UBC*	*TUBb*	*MDH*	*EF1a*	*18S*	*TUBa*	*UBQ*
(B)Ranking order under heat stress(better-good-average)											
Delta CT	*EF1b*	*ACT*	*18S*	*MDH*	*EF1a*	*CYP*	*TUBb*	*GAPDH*	*UBQ*	*TUBa*	*UBC*
BestKeeper	*TUBb*	*TUBa*	*MDH*	*ACT*	*18S*	*EF1b*	*GAPDH*	*CYP*	*EF1a*	*UBC*	*UBQ*
NormFinder	*EF1b*	*ACT*	*EF1a*	*18S*	*MDH*	*TUBb*	*GAPDH*	*CYP*	*UBQ*	*TUBa*	*UBC*
geNorm	*ACT / EF1b*		*MDH*	*18S*	*TUBb*	*CYP*	*EF1a*	*GAPDH*	*UBQ*	*TUBa*	*UBC*
Comprehensive Ranking	*EF1b*	*ACT*	*MDH*	*TUBb*	*18S*	*EF1a*	*TUBa*	*CYP*	*GAPDH*	*UBQ*	*UBC*
(C)Ranking order under NaCl stress(better-good-average)											
Delta CT	*ACT*	*CYP*	*EF1b*	*GAPDH*	*UBC*	*MDH*	*EF1a*	*UBQ*	*18S*	*TUBb*	*TUBa*
BestKeeper	*18S*	*MDH*	*CYP*	*EF1b*	*UBC*	*ACT*	*GAPDH*	*EF1a*	*UBQ*	*TUBb*	*TUBa*
NormFinder	*CYP*	*ACT*	*EF1b*	*GAPDH*	*EF1a*	*UBC*	*UBQ*	*MDH*	*18S*	*TUBb*	*TUBa*
geNorm	*ACT / EF1b*		*UBC*	*MDH*	*CYP*	*GAPDH*	*UBQ*	*EF1a*	*18S*	*TUBb*	*TUBa*
Comprehensive Ranking	*ACT*	*CYP*	*EF1b*	*MDH*	*UBC*	*GAPDH*	*18S*	*EF1a*	*UBQ*	*TUBb*	*TUBa*
(D)Ranking order under PEG stress (better-good-average)											
Delta CT	*ACT*	*UBC*	*EF1b*	*MDH*	*CYP*	*EF1a*	*UBQ*	*18S*	*GAPDH*	*TUBa*	*TUBb*
BestKeeper	*ACT*	*UBC*	*EF1b*	*CYP*	*MDH*	*TUBa*	*TUBb*	*18S*	*EF1a*	*GAPDH*	*UBQ*
NormFinder	*UBC*	*ACT*	*EF1b*	*CYP*	*MDH*	*EF1a*	*UBQ*	*18S*	*GAPDH*	*TUBa*	*TUBb*
geNorm	*ACT / EF1b*		*UBC*	*CYP*	*MDH*	*EF1a*	*UBQ*	*GAPDH*	*18S*	*TUBa*	*TUBb*
Comprehensive Ranking	*ACT*	*UBC*	*EF1b*	*CYP*	*MDH*	*EF1a*	*UBQ*	*18S*	*TUBa*	*GAPDH*	*TUBb*
(E)Ranking order under ABA treatment (better-good-average)											
Delta CT	*ACT*	*CYP*	*TUBb*	*EF1b*	*UBC*	*18S*	*MDH*	*EF1a*	*UBQ*	*GAPDH*	*TUBa*
BestKeeper	*MDH*	*UBC*	*ACT*	*EF1b*	*TUBb*	*CYP*	*18S*	*EF1a*	*GAPDH*	*UBQ*	*TUBa*
NormFinder	*ACT*	*CYP*	*EF1b*	*TUBb*	*18S*	*UBC*	*EF1a*	*UBQ*	*MDH*	*GAPDH*	*TUBa*
geNorm	*MDH / UBC*		*ACT*	*TUBb*	*CYP*	*EF1b*	*18S*	*EF1a*	*UBQ*	*GAPDH*	*TUBa*
Comprehensive Ranking	*ACT*	*UBC*	*MDH*	*CYP*	*TUBb*	*EF1b*	*18S*	*EF1a*	*UBQ*	*GAPDH*	*TUBa*
(F)Ranking order under GA treatment (better-good-average)											
Delta CT	*UBQ*	*EF1b*	*MDH*	*EF1a*	*UBC*	*ACT*	*GAPDH*	*TUBb*	*CYP*	*TUBa*	*18S*

Method/Rank	1	2	3	4	5	6	7	8	9	10	11
BestKeeper	*EF1b*	*MDH*	*UBC*	*ACT*	*UBQ*	*EF1a*	*GAPDH*	*CYP*	*TUBb*	*TUBa*	*18S*
NormFinder	*UBQ*	*EF1a*	*EF1b*	*GAPDH*	*MDH*	*ACT*	*UBC*	*TUBb*	*CYP*	*TUBa*	*18S*
geNorm	*MDH / UBC*		*ACT*	*EF1b*	*UBQ*	*EF1a*	*GAPDH*	*TUBb*	*CYP*	*TUBa*	*18S*
Comprehensive Ranking	*EF1b*	*UBQ*	*MDH*	*UBC*	*EF1a*	*ACT*	*GAPDH*	*TUBb*	*CYP*	*TUBa*	*18S*
(G)Ranking order under MeJA treatment (better-good-average)											
Delta CT	*ACT*	*CYP*	*TUBb*	*UBQ*	*MDH*	*GAPDH*	*18S*	*UBC*	*EF1b*	*EF1a*	*TUBa*
BestKeeper	*UBC*	*MDH*	*ACT*	*TUBb*	*CYP*	*EF1b*	*18S*	*GAPDH*	*UBQ*	*TUBa*	*EF1a*
NormFinder	*ACT*	*CYP*	*TUBb*	*UBQ*	*GAPDH*	*MDH*	*18S*	*EF1b*	*UBC*	*EF1a*	*TUBa*
geNorm	*ACT / MDH*		*UBC*	*TUBb*	*CYP*	*EF1b*	*UBQ*	*18S*	*GAPDH*	*EF1a*	*TUBa*
Comprehensive Ranking	*ACT*	*MDH*	*CYP*	*TUBb*	*UBC*	*UBQ*	*GAPDH*	*EF1b*	*18S*	*EF1a*	*TUBa*
(H)Ranking order under ETHYLENE treatment (better-good-average)											
Delta CT	*UBC*	*CYP*	*ACT*	*UBQ*	*MDH*	*TUBb*	*18S*	*EF1b*	*GAPDH*	*TUBa*	*EF1a*
BestKeeper	*MDH*	*UBC*	*ACT*	*EF1b*	*CYP*	*TUBb*	*GAPDH*	*UBQ*	*18S*	*TUBa*	*EF1a*
NormFinder	*CYP*	*UBC*	*UBQ*	*ACT*	*MDH*	*TUBb*	*18S*	*GAPDH*	*EF1b*	*TUBa*	*EF1a*
geNorm	*ACT / UBC*		*MDH*	*CYP*	*TUBb*	*EF1b*	*UBQ*	*GAPDH*	*18S*	*TUBa*	*EF1a*
Comprehensive Ranking	*UBC*	*ACT*	*CYP*	*MDH*	*UBQ*	*TUBb*	*EF1b*	*18S*	*GAPDH*	*TUBa*	*EF1a*
(I)Ranking Order of root (Better–Good–Average)											
Delta CT	*CYP*	*EF1a*	*GAPDH*	*TUBa*	*EF1b*	*ACT*	*UBC*	*UBQ*	*MDH*	*TUBb*	*18S*
BestKeeper	*18S*	*TUBa*	*CYP*	*EF1a*	*ACT*	*UBQ*	*GAPDH*	*EF1b*	*UBC*	*TUBb*	*MDH*
NormFinder	*EF1a*	*CYP*	*GAPDH*	*TUBa*	*EF1b*	*ACT*	*UBC*	*UBQ*	*MDH*	*TUBb*	*18S*
Genorm	*CYP / TUBa*		*EF1a*	*GAPDH*	*ACT*	*EF1b*	*UBC*	*UBQ*	*MDH*	*TUBb*	*18S*
Comprehensive ranking	*CYP*	*EF1a*	*TUBa*	*GAPDH*	*ACT*	*EF1b*	*18S*	*UBQ*	*UBC*	*MDH*	*TUBb*
(J)Ranking Order of stem (Better–Good–Average)											
Delta CT	*ACT*	*MDH*	*UBC*	*TUBb*	*EF1b*	*18S*	*GAPDH*	*CYP*	*UBQ*	*EF1a*	*TUBa*
BestKeeper	*UBC*	*MDH*	*TUBb*	*ACT*	*EF1b*	*CYP*	*18S*	*GAPDH*	*UBQ*	*EF1a*	*TUBa*
NormFinder	*ACT*	*TUBb*	*18S*	*MDH*	*EF1b*	*UBC*	*GAPDH*	*UBQ*	*CYP*	*EF1a*	*TUBa*
Genorm	*ACT / UBC*		*MDH*	*TUBb*	*EF1b*	*CYP*	*18S*	*GAPDH*	*UBQ*	*EF1a*	*TUBa*
Comprehensive ranking	*ACT*	*UBC*	*MDH*	*TUBb*	*EF1b*	*18S*	*CYP*	*GAPDH*	*UBQ*	*EF1a*	*TUBa*
(K)Ranking Order of leaf (Better–Good–Average)											
Delta CT	*CYP*	*EF1b*	*ACT*	*UBQ*	*EF1a*	*MDH*	*GAPDH*	*TUBb*	*18S*	*TUBa*	*UBC*
BestKeeper	*ACT*	*MDH*	*CYP*	*TUBb*	*EF1b*	*UBC*	*18S*	*EF1a*	*UBQ*	*GAPDH*	*TUBa*
NormFinder	*CYP*	*EF1b*	*ACT*	*EF1a*	*UBQ*	*GAPDH*	*MDH*	*TUBb*	*18S*	*TUBa*	*UBC*
Genorm	*ACT / MDH*		*EF1b*	*CYP*	*TUBb*	*UBQ*	*EF1a*	*GAPDH*	*18S*	*TUBa*	*UBC*

Method/Rank	1	2	3	4	5	6	7	8	9	10	11
Comprehensive ranking	*ACT*	*CYP*	*EF1b*	*MDH*	*UBQ*	*EF1a*	*TUBb*	*GAPDH*	*18S*	*UBC*	*TUBa*
(L)Ranking Order of all sample (Better–Good–Average)											
Delta CT	*ACT*	*EF1b*	*CYP*	*MDH*	*EF1a*	*GAPDH*	*UBQ*	*TUBb*	*18S*	*UBC*	*TUBa*
BestKeeper	*ACT*	*MDH*	*UBC*	*CYP*	*EF1b*	*TUBb*	*18S*	*UBQ*	*EF1a*	*GAPDH*	*TUBa*
NormFinder	*ACT*	*CYP*	*EF1b*	*MDH*	*EF1a*	*GAPDH*	*UBQ*	*TUBb*	*18S*	*UBC*	*TUBa*
Genorm	*ACT / MDH*		*EF1b*	*CYP*	*EF1a*	*UBQ*	*GAPDH*	*18S*	*TUBb*	*UBC*	*TUBa*
Comprehensive ranking	*ACT*	*MDH*	*EF1b*	*CYP*	*EF1a*	*UBQ*	*GAPDH*	*UBC*	*TUBb*	*18S*	*TUBa*

ΔCt algorithm analysis

The comparative ΔCt algorithm ranks the potential candidate reference genes expression stabilities based on the standard deviation values(**Supplemental Table 3**) [17]. As determined by ΔCt, *ACT* was consistently identified as the most stable gene through the total samples(**Supplemental Fig. 3**). *CYP* was the most stable reference gene in macadamia root and leaf, while *ACT* was the most stable reference gene in the stem in the different organs dataset from macadamia. In the subset of cold stress, NaCl stress, PEG stress, ABA treatment and MeJA treatment, *ACT* was still considered as the best stable gene in the 11 candidate reference genes, and *TUBa* was the least stable gene under the cold stress, NaCl stress, PEG stress, ABA treatment and MeJA treatment. Under the heat stress *EF1b* ranked top than *ACT* gene, and was determined the best stable reference gene. *UBQ* was ranked as the stable reference gene, while *18S* was the least stable reference gene under the GA treatment. In the ethylene treatment, *UBC* was the most highly ranked, and *EF1a* performanced the least stable gene(Fig. 2; Table 2, **Supplemental Table 3**).

geNorm Analysis

The geNorm program used the *M*-value to assess stability of the reference gene expression. The *M*-value was calculated at each step during stepwise exclusion of the least stable reference gene until two best gens were obtained[18]. The top two stable candidate reference gene were *ACT / MDH* for all samples(**Supplemental Fig. 4**), leaf and MeJA treatment, *ACT* and *EF1b* under cold stress, heat stress, NaCl stress and PEG stress, *MDH / UBC* in the GA treatment and ABA treatment, *ACT* and *UBC* for stem and ethylene treatment show the most stability. Additionally, as show **in** Fig. 2, Table 2 and **Supplemental Table 4**, the least stably expressed was *TUBa* under cold stress, NaCl stress, ABA treatment, MeJA treatment, stem and all samples, *UBC* under heat stress and leaf, *TUBb* under PEG stress, *18S* under the GA treatment and root, *EF1a* under the ethylene treatment.

NormFinder Analysis

The NormFinder algorithm ranking candidate reference gene stability based on a variance estimation approach[19]. The NormFinder ranking orders are similar to the geNorm analysis. According this analysis(**Figure S4**; Table 2; **Supplemental Table 5**), the most stable gene were *ACT* for cold stress, ABA treatment, MeJA treatment, stem and all samples, *EF1b* for heat stress, *CYP* for leaf, ethylene treatment and NaCl stress, *UBC* for PEG stress, *UBQ* for GA treatment, *EF1a* for root. (**Supplemental Fig. 5**)

BestKeeper Analysis

The BestKeeper algorithm determined the stabilities of candidate reference genes based on the CV ± SD values[20]. The stability rankings by BestKeeper was quite different from the ranking order determined by ΔCt, geNorm and NormFinder algorithms(**Supplemental Fig. 6**). *ACT* was considered as the best stable gene under all samples, PEG stress, and leaf. *TUBb* in heat stress, *UBC* for cold stress, MeJA treatment and stem subset, *18S* for NaCl stress and root, *MDH* for ABA treatment and ethylene treatment, *EF1b* for GA treatment were all ranked in top stable genes in different situation. Additionally, *18S* and *TUBa* were determined as the least stable reference gene in the most samples by BestKeeper algorithm(Fig. 2; Table 2; **Supplemental Table 6**).

Comprehensive Ranking

In order to obtain a consensus result of the best stable reference genes as recommended by the four above approached according to the RefFinder[21], the geometric mean of four algorithms corresponding rankings for each candidate gene were calculated(Table 2;

Fig. 2; **Supplemental Tables 7 and 8**). *ACT* was ranked the first stable gene in all samples, cold stress, NaCl stress, PEG stress, ABA treatment, MeJA treatment, stem tissue and leaf, *EF1b* for heat stress and GA treatment, *CYP* for root, *UBC* for ethylene treatment (Fig. 2; Table 2).

4. Validation of the reference genes with the *SAD* gene

To further validate the selected reference genes, the relative expression level of a target gene *SAD* in macadamia under different experimental conditions were evaluated using qRT-PCR. It was normalized using the most stable reference genes and two least stable reference genes as the internal control both singly and combination under different treatment subsets (Fig. 3). The relative expression abundance of *SAD* showed substantial divergence when normalized to different kinds of reference genes. In this study, the water treatment was designed as a negative control, when the most stable genes *ACT* and *CYP* were employed respectively and together to normalized expression levels of *SAD* gene, the expression abundant was not significantly effect; when the least stable genes *TUBa* were used to normalized expression level of target gene, the expression abundant was up-regulation dramatically. When the *EF1a* was used as the reference, the expression pattern of *SAD* gene was similar with the most stable genes normalization results. The expression level of *SAD* gene gradually declined under the cold stress using *ACT* and *EF1b* as the internal control, while the expression level was increased dramatically at 6h and 24h when the least stable reference genes *TUBa* was used, and increased sharply at 24h for *UBQ* normalized as internal reference. Under heat stress, the expression pattern of *SAD* gene were similar when the most stable reference genes (*ACT* and *EF1b*) and the least stable reference genes(*UBC* and *UBQ*) were used as controls, but the highest expression level at 12h was 2.5-fold higher by using *UBQ* normalizations than using the most stable reference gene *ACT* normalization. In PEG stress, the expression level of *SAD* gene was up-regulated at 24h and then down-regulated at 48h when using *ACT and UBC* genes normalization, while the expression appeared overestimated when normalized using *GAPDH* and underestimated for *TUBb*. Similarly, in NaCl stress, the expression profiles of the *SAD* showed same trends when stable reference genes *ACT* and *CYP* were used as internal control, when the least stable reference gene *TUBb* and *TUBa* were used as reference control, the expression level of target gene was significantly overestimated and underestimated respectively. Under ET and ABA treatment, the relative expression trends of *SAD* gene showed a similarly trend, appeared slightly descent at 6h and then increased at 24h when the most stable genes were used in combination and independent for normalization, while appeared down-regulated at 12h and 24h when normalized by using the least stable reference genes. In MeJA stress, a similar expression pattern was observed when either *ACT* or *MDH* was used alone and combination for normalization. While the expression levels of *SAD* gene were overestimated when the least stable genes *TUBa* and *EF1a* were used for normalization. In GA stress, the relative expression of *SAD* gene increased at 6h when using *EF1b* and *18S* as internal control, while declined slightly at 6h when normalized by using *TUBa* and *UBQ* as reference genes. Above all, the result showed that *ACT* and *EF1b* were more suitable for different treatment subset and *TUBa* failed standardized the expression data effectively.

In addition, to validate the selected reference genes, 4 genes involved fatty acid accumulation including *FATA*, *FAD*, *Oleosin* and *SAD* in qRT-PCR were compared with the expression pattern in RNA-seq results. In the qRT-PCR results, *Actin* was used as the normalizer to quantify the expression. As show in the Fig. 4, all the selected genes revealed similar expression file in the both methods. Therefore, our result provide the reliable reference gene in Macadamia.

Discussion

Gene expression quantification which as an important way for charactering gene function, has been accepted and applied widely in the field of genetic research[22]. qRT-PCR is commonly regarded as an practical method for accurate analysis of gene expression profiling with high sensitivity, specificity, reproducibility and less samples as MIQE(minimum information for publication of quantitative real-time PCR experiments, MIQE) a precondition[23]. There is no universally suitable reference genes under each experimental condition, and the reference genes expression is tissue-specific and various based on the physiological status of the organs or experimental condition[24–25]. Numerous studies have reported that the expression of housekeeping gene may be constant in some experiment condition, but varied considerably in other cases[26–28]. The nature protocols suggest that using a specifically and stably reference for normalization could ensure accurate results[29]. Therefore suitable reference genes selection and evaluation is critically mandatory for data normalization in gene expression analysis[30]. Suitable reference genes selection has been studied in the plant field recent years, including cucumber[31], *Setaria viridis*[32], sugarcane[33], wheat[34], soybean[35], *Brassica napus*[36], *Taxus spp.*[37], *Gentiana macrophylla*[38] *Botrytis cinerea*[39] and other various plants. To date, few studies have been carried out in macadamia involved in reference selecting. This has hindered the molecular functional studies of important genes involved in stress

conditions and specific organisms. In present study, we systematically validated the stability of 11 traditional reference gene under the specific environmental condition or treatment as well as the in different tissues of *Macadamia integrifolia* seedlings. Traditional housekeeping genes involved in cytoskeleton structure(*ACT*, *TUBa*, *TUBb*), protein synthesis (*EF1a*, *EF1b*, *18S*), biological metabolic process(*GAPDH* and *UBQ*), and multifunctional proteins (*CYP* and *MDH*), are selected and the open reading frame sequences of these genes were cloned from *Macadamia integrifolia*.

It is recommended that a comprehensive RefFinder algorithms could provide a reliable assessment of the expression stability of candidate reference gene under various experiment condition for real-time qRT-PCR analysis[40]. This statistical algorithms four methods, includingΔCt, geNorm, NormFinder and BestKeeper, that have been developed to assess the expression stability of candidate reference genes for accurate normalization in gene expression studies were used to rank the reference gene. The ranking results by different algorithms are relatively coincident mostly. In the heat treatment subset, *EF1b* and *ACT* are top two genes inΔCt, NormFinder and geNorm analysis, and ranked third and fourth in bestkeeper analysis. In ABA treatment, NaCl, stem, and all samples sets, *TUBa* was always ranked the least stable gene by four different algorithms. But sometimes the stability ranking showed significant differences due to the complementary different statistical program (Table 2). For example, under NaCl stress, *ACT* was ranked first by ΔCt and geNorm, while it was ranked sixth by BestKeeper. *CYP* was ranked first by NormFinder, and followed by *ACT.* The ΔCt method by comparing relative expression of pairs of genes within each sample to confidently identify stability of reference genes[17]. geNorm is one of the most commonly used method for systematic validation procedures of reference gene stability[18]. geNorm identifies two reference genes according to the similarity of expression profile in each sample and the lowest intra-group variation [41–42], and not suitable for distinguishing the gene of similar expression pattern. Andersen developed the NormFinder approach[19], which takes both the iner- and intra-group variations into account, and combines them into a stability value, and finally ranks the top genes with minimal inter- and intra-group variation. Most of time the results of NormFinder is similar to the geNorm results. For instance in the heat stress samples, *EF1b* and *ACT* were ranked top two stable reference genes, and *TUBa* and *UBQ* were the least stable reference genes, other genes ranked order have slightly change by geNorm and NormFinder. The BestKeeper software analysis is not restricted to the stability of reference genes but also target gene[20], it determines the stability ranking of the reference genes according to their SD and CV values[43–44]. Compare with geNorm and NormFinder, the BestKeeper results have significant difference owing to the different calculation strategy. For example, in the subset of all samples, *ACT* was ranked first genes by all the programs. Under cold stress, *ACT* was determined the most stable genes byΔCt, geNorm, NormFinder and comprehensive ranking, excepted BestKeeper. As these programs ranked different most stable reference genes [45–47], we selected the most reliable reference gene by using a comprehensive tool RefFinder, which that rank the reference genes based on the geometric mean of the weights of every gene calculated by each program[21]. The comprehensive results obtained on the basis of statistical analysis byΔCt, geNorm, NormFinder and BestKeeper showed mostly consistency in this study.

ACT is commonly known to be a reliable reference gene[48]. Although there are studies have provided evidence that *ACT* vary considerably in *Zea mays* [49], our finding support that *ACT* is the most stable reference gene for the relative quantification in Macadamia among all samples, cold stress, salt stress, PEG stress, ABA treatment and MeJA treatment samples. *Elongation factors* involved in translocation of aminoacyl-transfer RNA to the ribosome during protein synthesis. *EF1a* and *EF1b* are highly conserved in sequence and expression in eukaryotes, usually used as internal genes. *EF1b* was established as the most appropriate reference gene under GA treatment and heat treatment in this study, while *EF1a* is considered the most stable gene under complex developmental process in *Populus* [50]. *UBC* ranked first than *ACT* gene in ethylene treatment which consistent with the result in *platycladus orientali* [43]. *CYP*(*Cyclophilin*), a specific cytosolic binding protein, which is ranked the top stable gene in root in macadamia. *TUBa* mostly was not satisfactory for qRT-PCR analysis in present study, but it was sufficiently stable in *Hererosigma akashiwo* (Raphidophyceae) [51], which has been selected as reference gene in *Ulva linza* [52] and the diatom *Pseudo-nitzschia multistriata*[53].

Our previous studies found thatΔ^9-Stearoyl-ACP desaturases(SAD) is the key of unsaturated fatty acid synthesis and accumulation in macadamia oil (no publish data). The expression profile of *SAD* and the fatty acid level were affected in Arabidopsis Crown Galls under drought and hypoxia stress conditions[54]. In this study, we quantified the *SAD* gene expression using two most stable reference genes both singly and combined as well as two least stable genes as an internal control in qRT-PCR analysis to validate the selected reference genes in macadamia. When the least stable genes *TUBb* was validated as a reference gene for normalization, the expression pattern of *SAD* gene was obviously overestimated, and underestimated using *TUBa* to reference under the NaCl treatment (Fig. 3). The *SAD* gene expression was induced using the stable reference gene, while down-regulation when normalization with the two least reference gene *TUBa* and *EF1a* after the 6h ET treatment. So these results demonstrated that the suitable selection of internal control is critically important for the normalization, and used reference without validation would be leading misinterpretation.

RNA-seq is an approach to analysis the transcriptome profiling of various species, and recently was used to search for candidate reference genes. In this study, the target gene expression profile consistent both in qRT-PCR results and RNA-seq results, and they supported each other. So, our finding indicated that the result of this experiment are credible. The reference genes evaluated in this study would be very useful for further gene expression analysis in molecular mechanism of fatty acid accumulation under different treatment in Macadamia.

Conclusions

The reference genes selected in current study will be helpful for accurate normalization of qRT-PCR data in *M. integrifolia*. In this study, we selected the most stable reference genes in different tissue of macadamia seedlings and under different treatment. We found that *ACT* shows a good stability in all samples. *UBC* could be used to normalization when treated by ethylene. In the GA treatment samples, *EF1b* is the best reference genes. The identification of the suitable reference genes in this study will facilitate the future work on gene expression studies in Macadamia to improve our standing on the molecular mechanisms of fatty acid synthesis and accumulation under different experiment condition.

Methods

Plant materials and stress treatments.

NanYa No1, a macadamia cultivar developed by the South Subtropical Crops Research Institute of China Academy of Tropical Agricultural Sciences(CATAS). The Seeds of cultivars NanYa No1 were provided by National Field Genebank for Tropical Fruits of the South Subtropical Crops Research Institute，Guangdong Province and Chinese Academy of Tropical Agricultural Sciences, Zhanjiang, China(110º27'E; 21º17'N). Macadamia seeds were sowed and maintained in a glass bottle with 1/2 SD medium(without hormone) kept at 20°C±2 . When the seedlings stem of macadamia sizes were 2.1-3.3 mm in diameter, different treatment could be conducted in this study.

For experiments, each treatment group was set up in triplicate. Control group was treated with water. The organ-specific series of samples (root, stem, leaf) were collected from the seedlings. For drought treatment, 20% PEG-6000 solution(w/v, polythyleneglycol) was applied to incubate the plants for 0, 12, 24, 48 and 72h. For cold and heat stress, plants in the pots were placed at chamber with the temperature of 4°C and 42°C respectively for 0, 2, 6, 12 and 24h. For salinity treatment, seedling were transferred to 1/2 SD medium with 150 mmol NaCl for 0, 12, 24, 48, 72h. For hormone treatment, plants were imposed in 100 µmol/L methyl jasmonate(MeJA), abscisic acid(ABA), gibberrellins(GA) or ethylene (ETH) for 0, 2, 6, 12 and 24h. After that, the roots, leaves and stems were sampled separately at different periods for each treatment used for expression analysis. The detailed information of samples collected from various tissues/experimental conditions were also listed in **Table S1**. All samples were frozen in liquid nitrogen and then stored at -80°C prior to RNA isolation.

Total RNA Isolation and cDNA Synthesis

The frozen samples were ground to a fine powder in liquid nitrogen using a pestle and mortar. The total RNA was extracted from the samples using Plant Total RNA Isolation Kit Plus (Cat. No.RE-05021/05022/05024, FOREGNE, China) following the manufacturer's recommendation. RNA purity and concentration were measured by NanoDrop spectrophotometer (Thermo, Germany), and RNA integrity was verified by agrose gel electrophoresis with ethidium bromide staining. First-stand cDNAs were synthesized from 1µg total RNA with a final volume of 20 µL using the RTIII all-in-one Mix (Monad, China). The cDNA synthesis mixture was incubated at 37°C for 2 minutes and then 55°C for 15 minutes. This reaction was terminated by incubating it at 85°C for 10 seconds. The cDNAs were diluted 1:50 with nuclease-free water before use in subsequent experiments.

Selection of Candidate Reference Genes and Primer Design

Eleven classical housekeeping genes commonly used as internal control for expression studies. The open reading frame sequence of these genes were cloned from macadamia（GenBank accession number MN650750, MN627205, MN627206, MN627207, MN627208, MN627209, MN627210, MN627211, MN627212, MN627213, MN627214）. The candidate reference genes were listed in **table 1** with their respective reference where they were first described. The amplification primers for real-time PCR were designed using Primer-BLAST in NCBI. The criteria for primer design were set as follows: primer lengths of 20-24 bp, GC contents of 45-55%, melting temperature(Tm) in a range of 55-60°C and amplification lengths of 100-250 bp.

Real-Time Quantitative Polymerase Chain Reaction

qRT-PCR was conducted in 96-well plates and performed on the LightCycler 480 (Roche Molecular Biochemicals, Mannheim, Germany). The reaction mix contained 1μL diluted cDNA5μL Tap SYBR Green qPCR Premix (Nova, Jiangsu, China), 0.5MlμL the specific primer pairs, and 3.5μL ddH2O in a final volume of 10μL. Three biological replicates for all of the samples and three technical replicates of each biological replicate were carried out. The qRT-PCR protocol was follows: 95°C for 3 min, 45 cycles of 95°C 10 s, 60°C for 30 s, 72°C for 10s. To verified the specificity of each primer, a melting-curve analysis was include. The mean amplification efficiency of each primer pair was checked by the LinRegPCR program[55].

Analysis of real-time PCR data

The comparative ΔCt algorithm assesses the expression stability of reference genes by the mean standard deviation values from the test sample[17]. The ΔCt value indicated the variably transcription of candidates.

The geNorm VBA applet determines the best reference based on the geometric mean and pair wise variation of each gene from all the candidate reference genes in the total sample. Two parameters were used to evaluate the expression stability of the reference genes; the average expression stability value(M value), based on the pairwise variation between a particular gene compared to all others, and the pairwise variation(Vn/n+1), which determines the required number of genes to result in a more accurate normalization[18]. A cut-off value of Vn/n+1 <0.15 indicates that an additional reference gene makes no significant contribution to the normalization.

While geNorm performs a stepwise exclusion of the least stably expressed genes, NormFinder uses a model-based approach, which calculates both inter- and intra-group variability to estimate the stability of gene expression. NormFinder identified the optimal normalization genes among a panel of candidates according to their expression stability in a given sample set or given experimental designs. This algorithm evaluates not only the overall expression variation of the candidate reference gens, but also the variation between subgroup of samples. In addition to data from real-time PCR , NormFinder can also analyze expression data obtained through other quantitative gene expression methods, such as microarray. Ranking the stability based on the stability value (SV) where lower stability value represents higher gene expression stability and vice versa[19].

Bestkeeper is used to determinate the stability of reference genes based on the coefficient of variance (CV) and the standard deviation(SD) of the average Ct values. The lower of CV±SD values, the more stable the candidate reference genes, and gene with SD >1 would be considered unacceptable and should be excluded[20].

RefFinder is a user-friendly web-based comprehensive tool developed for evaluating and screening reference genes from extensive experimental datasets. It integrates the currently available major computational programs (geNorm, Normfinder, BestKeeper, and the comparative ΔCt method) to compare and rank the tested candidate reference genes according to the geometric mean of four algorithms corresponding rankings. [21]. We analysis the qRT-PCR data on the RefFinder mirror site(http://blooge.cn/RefFinder/) .

Evaluating Reference Genes Expression

The expression level of the target gene *SAD* was analyzed using the most stable and least stable reference genes after normalization across all the experimental sets. The amplification efficiencies of the target genes were also estimated by the LinRegPCR program. The average Ct value was calculated from three biological and technical replicates and used for relative expression analyses. The relative expression data were calculated according to the qRT-PCR $2^{-\Delta Ct}$ method and presented as fold change[56].

Abbreviations

SADΔ9-Stearoyl-ACP desaturases

18S18S ribosome RNA

ACTActin

CYPCyclophilin

EF1a Elongation factor 1-α

EF1b：Elongation factor 1-β

GAPDH：Glyceraldehyde-3-phosphate dehydrogenase

MDH：Malate dehydrogenease

UBC：Ubiquitin-conjugating enzyme

TUBa：Tubulin-α

TUBb：Tubulin -β

UBQ：Ubiquitin-A

FATA：fatty acid thioesterase

FAD：fatty acid desaturation

MeJA：jasmonate

ABA：abscisic acid

GA ：gibberrellins

ETH：ethylene

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

This manuscript has been reviewed and approved by all authors.

Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Q.Y and Z.P.Y contributed equally to this work. Q.Y performed the experiments, prepared the figures and tables, and drafted the manuscript. Z.P.Y participated in design of the experiments and performed the statistical analysis. Y.L.Z prepared the plants and performed part experiments. H.Z and M.H.Z provided the Macadamia tree of NanYa No1 cultivars to collect Macadamia seeds. H.L revised the manuscript. All authors have read and approved the manuscript.

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Figures

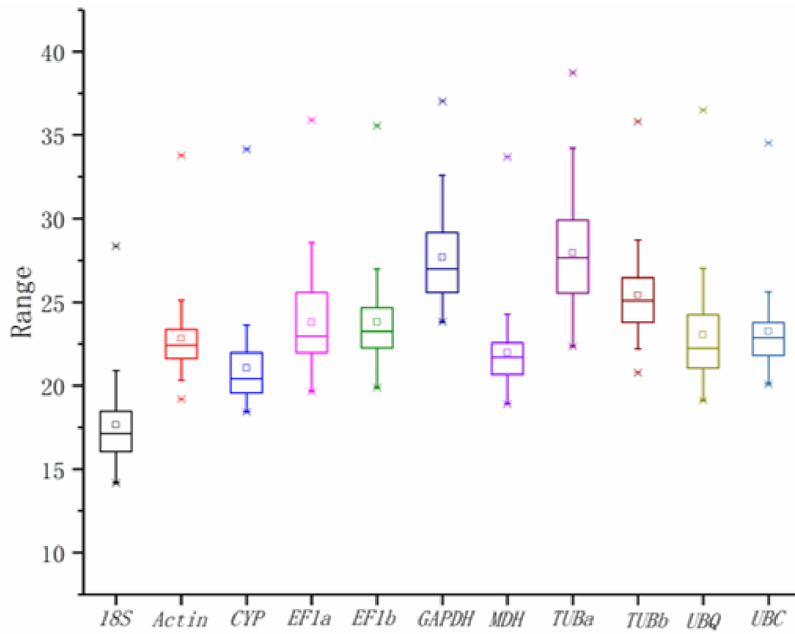

Figure 1

Distribution of qRT-PCR Ct values of candidate reference genes across all samples. The final Ct value of each samples was the mean of three biological and technical replicates. Box graph represents the interquartile range. The line across the box is the median value. The two bars represent the minimum and maximum values respectively; the small square in the box show the mean values. * represents the non-outlier and outlier.

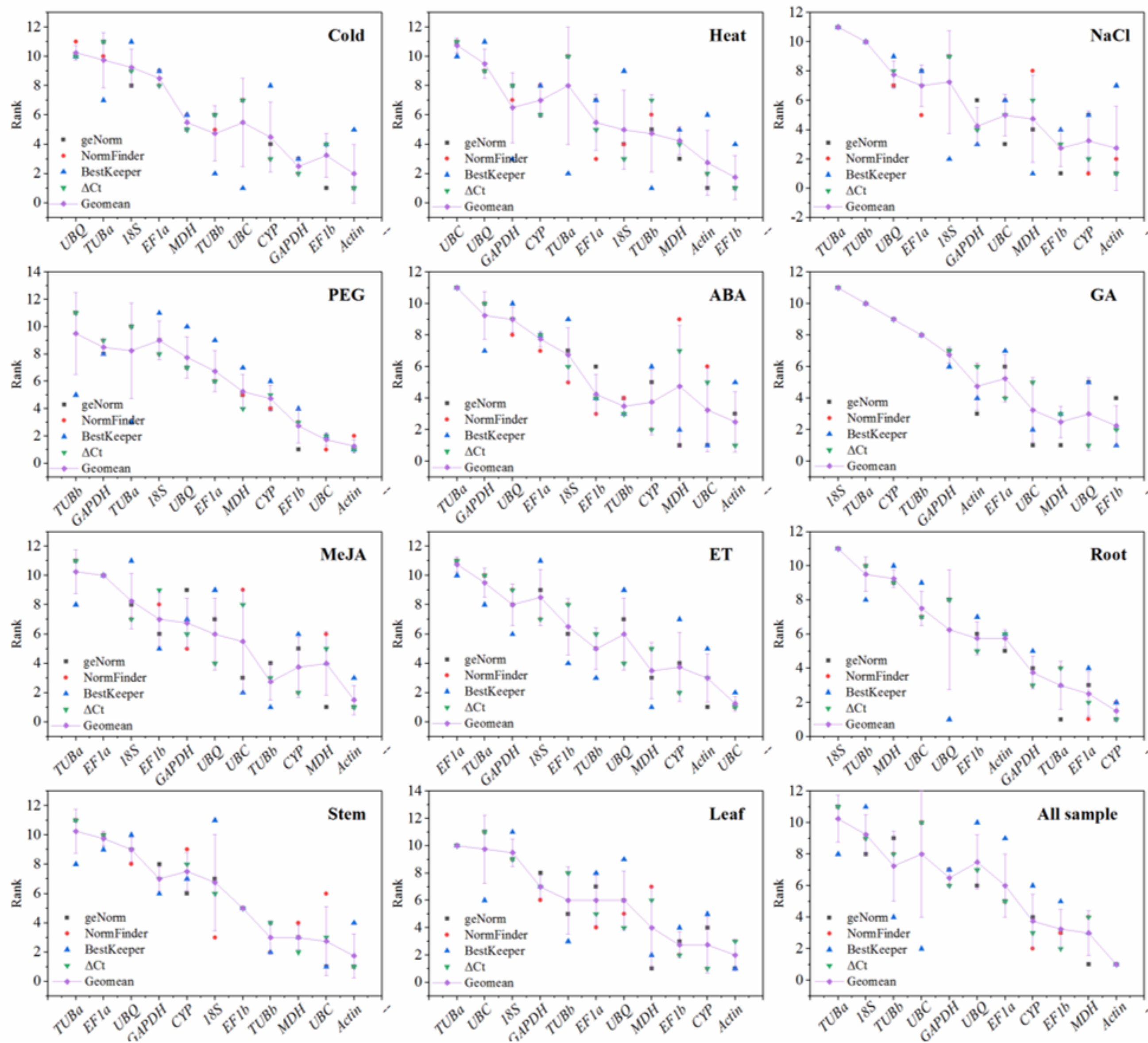

Figure 2

Aggregation of four rankings. In each plot, it contain five types of ranking including (geNorm, NormFinder, BestKeeper,ΔCt and Geomean). The geometric ranking of candidate genes was calculated comprehensively by four types of rankings (geNorm, NormFinder, BestKeeper and ΔCt) under different treatment.

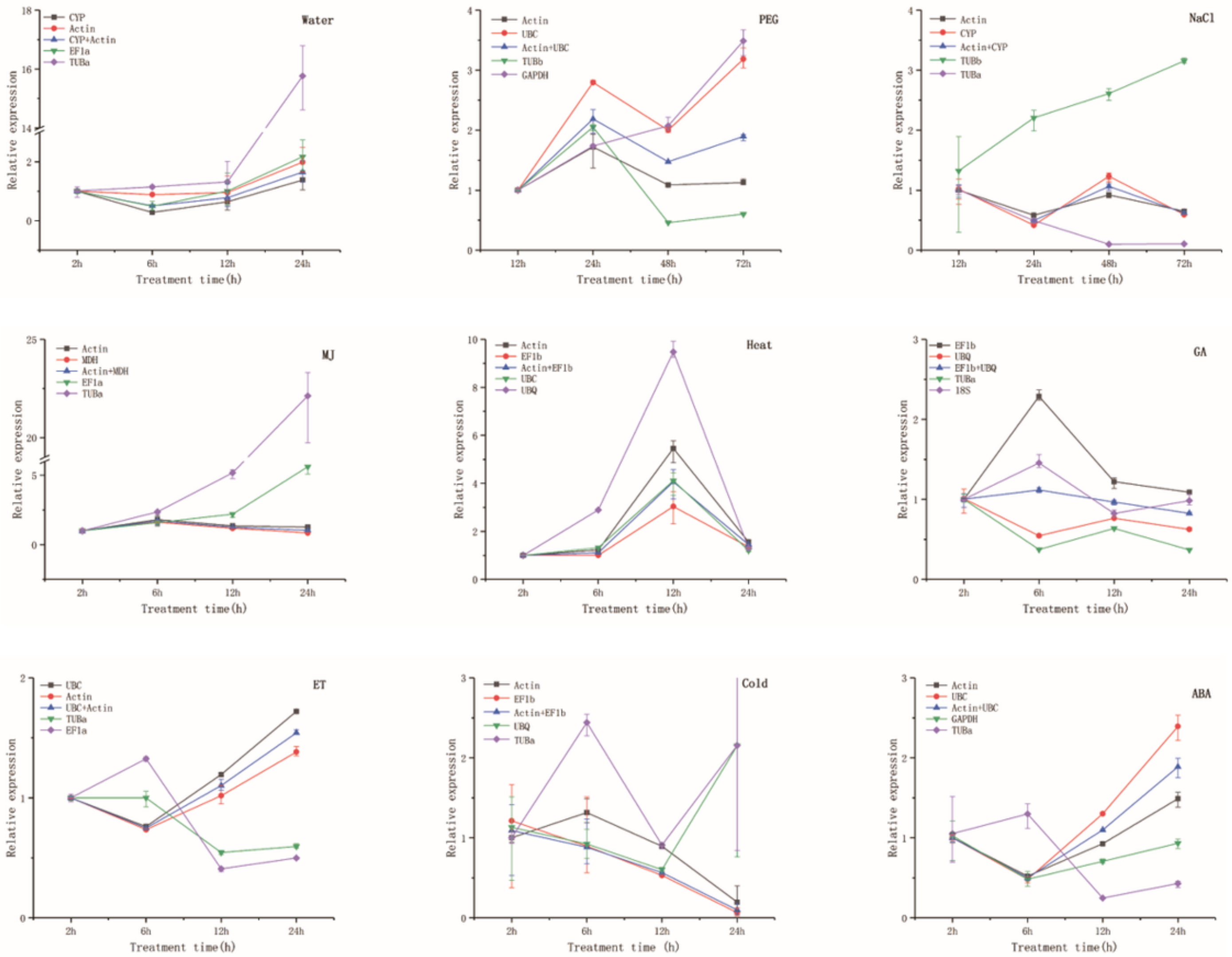

Figure 3

Relative quantification of SAD expression using the validated reference gene(s). The broken line graph represent the relative expression trend when compared to non-treatment samples under different tissue and treatment. cDNA samples were taken from the same subset used for gene expression stability analysis. Roots, stem, leaves were collected from Macadamia integrifolia seedling subjected to various treatment.

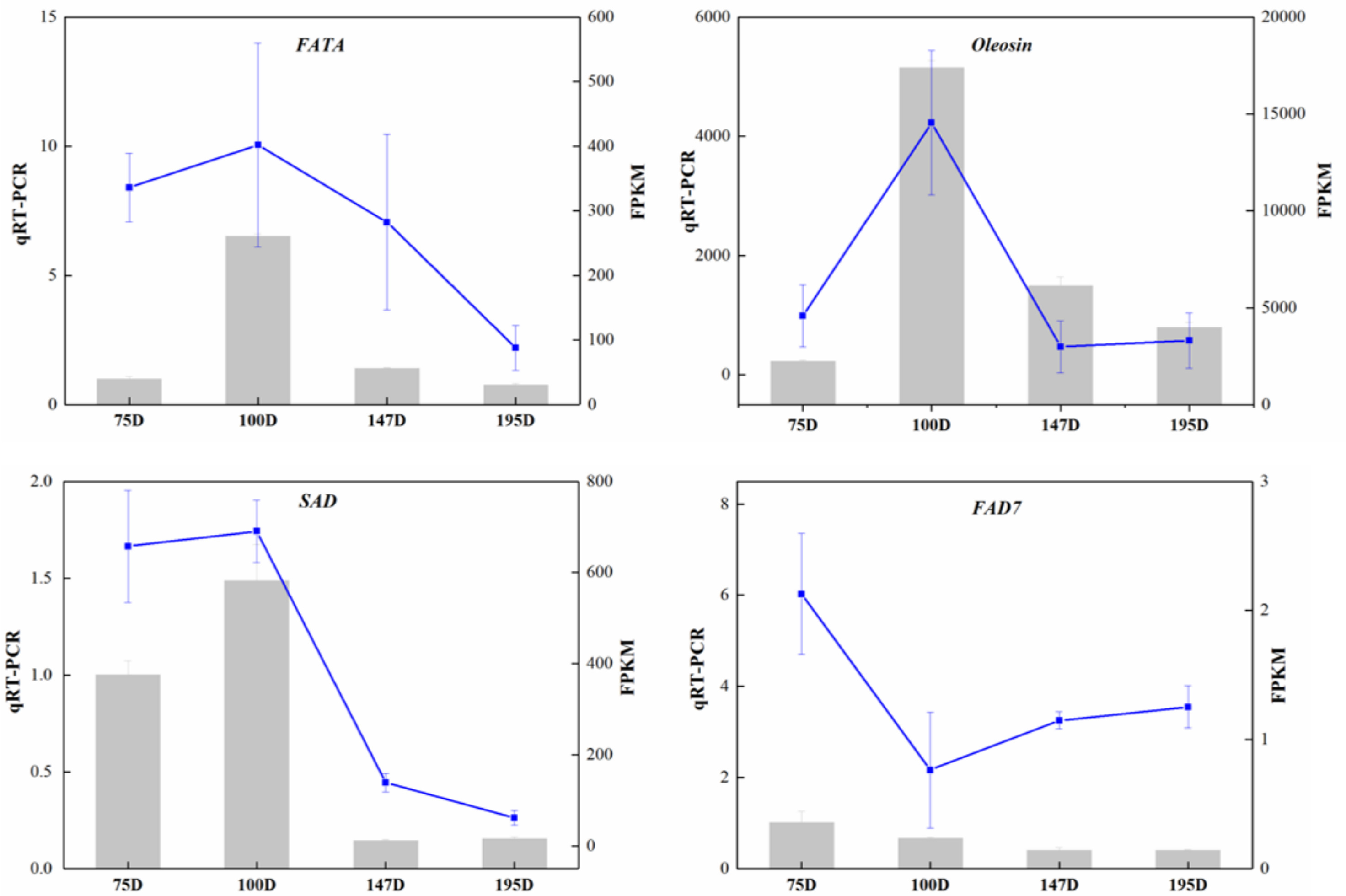

Figure 4

Validation of qRT-PCR results through comparison with RNA-seq expression profiles. The histograms show the qRT-PCR results involved fatty acid biosynthesis in kernel of Macadamia after anthesis; the line charts show the FPKM values of these fatty acid biosynthesis genes in kernel of Macadamia. qRT-PCR results represent the mean(± SD)of three biological replicates. 75D (75 day after anthesis), 100D (100 day after anthesis), 147D (147 day after anthesis)195D (195 day after anthesis).

Supplementary Files

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