Validation of reference genes for gene expression studies in tartary buckwheat (*Fagopyrum tataricum* Gaertn.) using quantitative real-time PCR

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Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) is a sensitive technique for quantifying gene expression levels. By implementing three distinct algorithms (geNorm, normFinder and BestKeeper), we have validated the stability of the expression of seven candidate reference genes in tartary buckwheat , including *FtSAND*, *FtCACS*, *FtExpressed1*, *FtGAPDH*, *FtActin*, *FtEF-1a* and *FtH3*. In this study, the results indicated that *FtCACS* and *FtSAND* were the best reference genes for 'abiotic cotyledons', and *FtExpressed1* and *FtEF-1α* were the best reference genes for aluminium treatment; *FtCACS* and *FtExpressed1* performed the best for the immature seed stage; *FtCACS* was best for the abiotic treatment, and *FtH3* appeared to be the most suitable reference gene for the abiotic treatment in hypocotyls and all samples in this study. In contrast, *FtActin* and *FtGAPDH* are unsuitable genes. Our findings offer additional stable reference genes for gene expression research on tartary buckwheat at the immature seed stage and under abiotic treatment.

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Abstract

16   Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) is a

17   sensitive technique for quantifying gene expression levels. By implementing three distinct

18   algorithms (geNorm, normFinder and BestKeeper), we have validated the stability of the

19   expression of seven candidate reference genes in tartary buckwheat, including *FtSAND*, *FtCACS*,

20   *FtExpressed1*, *FtGAPDH*, *FtActin*, *FtEF-1a* and *FtH3*. In this study, the results indicated that

21   *FtCACS* and *FtSAND* were the best reference genes for 'abiotic cotyledons', and *FtExpressed1*

22   and *FtEF-1α* were the best reference genes for aluminium treatment; *FtCACS* and *FtExpressed1*

23   performed the best for the immature seed stage; *FtCACS* was best for the abiotic treatment, and

24   *FtH3* appeared to be the most suitable reference gene for the abiotic treatment in hypocotyls and

25   all samples in this study. In contrast, *FtActin* and *FtGAPDH* are unsuitable genes. Our findings

26   offer additional stable reference genes for gene expression research on tartary buckwheat at the

27   immature seed stage and under abiotic treatment.

Introduction

28

29   Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) has become

30   the most prevalent quantification method used in assays of gene expression on account of its

31   specificity, accuracy, efficiency and high sensitivity (Jiang et al. 2014). The data from qRT-PCR

32   can be analysed using absolute or relative quantification. Absolute quantification yields an exact

33   gene copy number by transforming quantification cycles (Hruz et al.) into a standard curve.

34   Relative quantification relies on internal control genes as references to present the qRT-PCR data

35   of the target genes (Ginzinger 2002). Relative quantification has become a more widely used

36   method in gene expression assays because most researchers mainly focus on the analysis of

37   differences in gene expression (Wu et al. 2016). To normalize qRT-PCR data, a reference gene is

38   needed, and it should be universally valid, with a similar expression level across all feasible

39   cells, tissue samples and experimental treatments (Huang et al. 2014). However, ideal reference

40   genes do not actually exist (Remans et al. 2014). The selection of the appropriate reference gene

41   is a critical step in controlling the variability of samples when using a sensitive qRT-PCR

42   technique (Tong et al. 2009). If the reference gene is appropriate, the discrepancies that may

43   exist in terms of the initial sample amount, RNA integrity, RNA recovery, and the efficiency of

44   cDNA synthesis will be eliminated. Statistical algorithms, such as geNorm (Vandesompele et al.

45   2002), normFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004), have been

46   developed to help to select appropriate reference genes.

47   Most studies about gene expression in bacteria (Wu et al. 2017), yeast (Cankorur-Cetinkaya et

48   al. 2012) and mammals (Terzi et al. 2010) now include reference gene validation (Chapman &

49   Waldenstrom 2015). However, the validation of reference genes in plants has received very little

50   attention, and housekeeping genes tend to be used as references without any appropriate

51   validation (Gutierrez et al. 2008). In 2005, Czechowski et al were the first to present a list of

52   stably expressed Arabidopsis genes under a large range of experimental conditions, and the

53   evidence clearly showed that several genes are expressed more stably than traditional reference

54   genes (Czechowski et al. 2005). Since then, suitable reference genes for gene expression studies

55   have been reported in higher plants such as flax (Huis et al. 2010), soybean (Hu et al. 2009),

56   tomato (Lovdal & Lillo 2009), *Pisum sativum* (Die et al. 2010), *Brachypodium distachyon* (Hong

57   et al. 2008) and carrot (Tian et al. 2015). However, reference gene stability is not consistent

58 across experimental conditions and plant species. Consequently, it is necessary to find additional

59 reference genes for different conditions and species.

60   Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) belongs to the Polygonaceae family (Kim

61 et al. 2013), and as an important functional food material, it has a relatively high flavonoid

62 content. Tartary buckwheat is capable of thriving in regions with poor soil or harsh climates

63 (Kim et al. 2009). The results of genome sequencing also show that tartary buckwheat has a

64 remarkable ability to cope with highly variable environmental stress, including drought, salinity,

65 UV-B and cold (Zhang et al. 2017). The stress resistance of tartary buckwheat is mainly due to

66 its abundant flavonoids (Suzuki et al. 2005). In particular, tartary buckwheat is a naturally

67 aluminium (Al) tolerant species (Wang et al. 2015). Abiotic stresses and flavonoid metabolism

68 regulate the expression of these genes in plants at both transcriptional and post-transcriptional

69 levels. There are many reports on tartary buckwheat under various experimental conditions in

70 which gene expression is normalized to a reference gene (*H3*) for semi-quantitative RT-PCR or

71 qRT-PCR (Bai et al. 2014). However, to date, there is no systematic strategy to analyse tartary

72 buckwheat reference genes at the immature seed stage or under abiotic stress.

73 In this work, we aimed to evaluate the potential use of different reference genes for internal

74 normalization to more accurately measure the expression level of genes of interest in tartary

75 buckwheat. Seven candidate reference genes were selected, and the stability of their expression

76 was assessed in tartary buckwheat at the immature seed stage and under different abiotic stress

77 treatments. Evaluating the stability of the expression of candidate reference genes depends on

78 statistical analysis. Three different statistical software programs (geNorm, normFinder, and

79 BestKeeper) were used to calculate the variability of the expression of the candidate genes and

80 determine which were the most suitable.

81

Materials & Methods

Plant materials and treatments

84   Tartary buckwheat ("Xiqiao No. 2") seeds were grown in the field on a farm (XTBG; 29°59′N,

85 102°59′E; 800 m elevation) at Sichuan Agricultural University, Ya'an, Sichuan, China (Li et al.

86 2012). Tissues, including roots, stems, leaves, flowers, immature seed 1 (seed formation started)

87 and immature seed 2 (seeds in the milk) were collected at the immature seed stage (Gupta et al.

88 2011). The 7-day-old seedlings were stressed with saline or drought by adding 100 mM NaCl or

89   20% PEG 2000, respectively, to the medium. The 7-day-old seedlings were exposed in a

90   chamber at 4℃ with a 16 h photoperiod for cold treatment (Gao et al. 2016). UV-B treatment

91   was conducted under UV-B (302 nm, 0.1 mW/cm^2) in a chamber . After 0, 2, 4, 6, 12 and 24 h of

92   treatment, all stressed seedlings were collected and separated into cotyledons and hypocotyls.

93   For the Al treatment, the samples were processed according to a previous report (Zhu et al. 2015).

94   Root tips (0-2 cm) and basal roots (2-4 cm) were sampled under both −Al and +Al conditions.

95   All samples were collected in two biological replicates, and RNA was extracted immediately.

96   **Total RNA isolation and cDNA synthesis**

97   Total RNA was isolated from various samples with an RNAout 2.0 kit (Tiandz, China)

98   according to the manufacturer's instructions. To remove trace DNA from samples, total RNA

99   extractions were treated with RNase free DNase I. The RNA integrity was detected using 2%

100   agarose gels. A Bio-RAD smart spec^{TM}plus spectrophotometer was used to determine the RNA

101   purity. cDNA was synthesized with a PrimeScript^{TM} RT reagent kit and gDNA Eraser (Perfect

102   Real Time) (TaKaRa, Dalian, China).

103   **Selection of candidate reference genes and design of qRT-PCR primers**

104   Potential homologues of the seven candidate genes were identified from the transcriptome

105   sequencing data of tartary buckwheat ('Xiqiao No. 2') (Yao et al. 2017). Primers were designed

106   using Primer Premier 5.0. All primers used in this research are listed in Table 1. The specificity

107   of the amplification was assessed based on the presence of a single band of the expected size in a

108   1.5% agarose gel following electrophoresis and a single peak in the qRT-PCR melting curve.

109   **Quantitative real-time PCR**

110   The qRT-PCR procedure was designed in accordance with MIQE guidelines (Bustin et al.

111   2009). qRT-PCR was executed in a CFX96 Real Time PCR system (Bio Rad, Singapore) with a

112   SYBR Premix EX *Taq* kit (TaKaRa, Japan) in a total reaction volume of 15 μL that included 10

113   μL of SYBR Green mix, primers at 0.5 μM each and 1 μL of cDNA. The amplification

114   conditions were as follows: 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 20 s. A

115   melting curve from 60 to 95°C was used to verify the specificity of the PCR amplification. All

116   genes were amplified from cDNA. Ten-fold serial dilutions of cDNA samples from young leaves

117   were used to establish the standard curves to calculate the amplification efficiency of each primer

118   pair.

119

120   **Statistical analysis**

121   Three software programs (geNorm v3.5, the Excel add in of normFinder v0.953 and

122   BestKeeper v1) were used to analysis the stability of reference gene expression across all

123   experimental sets (Andersen et al. 2004; Pfaffl et al. 2004; Vandesompele et al. 2002). SPSS

124   v17.0 was used to calculate the span of Cq values for each gene by drawing a box-whisker plot,

125   and the expression levels of *FtSTAR* and *FtDFR* were showed by mean ± standard deviation

126   (SD).

127

128   # Results

129   **RNA solution and quality**

130   A series of 58 samples from tartary buckwheat were divided into six different groups. 'Abiotic

131   cotyledons' was composed of cotyledons from all stress-treated samples. 'Abiotic hypocotyls'

132   included hypocotyls from all stress-treated samples. 'Abiotic total' was composed of 'abiotic

133   cotyledons' and 'abiotic hypocotyls'. 'Al treatment' comprised all samples that were treated with

134   Al. 'Immature seed stage' consisted of six tissues (roots, stems, leaves, flowers, immature seed 1

135   fand immature seed 2) at the immature seed stage based on the state of the seed. Finally, 'total'

136   included all the samples in this study. Protein and organic pollutants were isolated and removed

137   from all samples via RNA extraction. The total RNAs, with A_{260}/A_{280} ratios of 1.8-2.0, were

138   reverse transcribed into cDNA as templates for qRT-PCR detection.

139   **Expression profiles of candidate reference genes**

140   We selected the best reference genes (among the 7 candidate genes) from the seven candidate

141   genes in six different groups (sequencing data was shown in Supplemental Fig. S1). The

142   amplification efficiencies (E) of all reactions ranged from 95.5% to 107.5% and were calculated

143   from standard curves with good linear relationships (R^2>0.99). Single-peak melting curves were

144   obtained for all qRT-PCR amplifications (Supplemental Fig. S2). A simple and commonly used

145   method to identify stably expressed genes is to compare the span of quantification cycle (Hruz et

146   al.) values for each gene in the qRT-PCR reactions. The results showed that the candidate

147   reference genes spanned a wide range of Cq values, ranging from 14.98 *(FtGAPDH)* to 29.65

148   *(FtActin)*, with the median Cq values of the genes ranging from 17.59 (*FtGADPH*) to 25.5

149   (*FtSAND*). The reference gene expression levels, in descending order, were *FtGAPDH, FtEF-1,*

150   *FtH3, FtCACS, FtActin, FtExpressed1* and *FtSAND*. *FtActin* (median, 23.55; Cq variation,

151   11.38) showed the most variability, and the *FtH3* gene (median, 20.19; Cq variation, 3.67)

152   showed the least variability (Fig. 1).

153   **Software: geNorm, NormFinder and BestKeeper**

154   After a simple comparison of the raw Cq values, the three software programs were used to

155   further analyse the stability of the expression of the seven candidate genes. The genes were

156   ranked in descending order in terms of the stability of their expression in each of six groups

157   (Table 2), and the details of the cotyledons and hypocotyls under abiotic stress are provided in

158   Supplemental Table S1. GeNorm performed a stepwise exclusion of the most unstable gene and

159   then recalculated M until only two genes remained, and these two genes had the most stable

160   expression (Vandesompele et al. 2002). The gene with the lower M value was considered to have

161   the most stable expression. An M value limit of <1.5 was suggested by geNorm. All the results

162   from geNorm were lower than 1.5 in 'abiotic cotyledons' and 'total'; *FtCACS* and *FtH3* (M =

163   0.33, 0.60) were chosen as the most stable genes, while *FtH3* and *FtSAND* (M = 0.65, 0.55) were

164   identified as the most stable genes in the 'abiotic hypocotyls' and 'abiotic total' groups,

165   respectively, In 'immature seed stage', *FtCACS* and *FtExpressed1* (M = 0.16) were recognized

166   as the most stable genes. Finally, *FtEF-1α* and *FtExpressed1* (M = 0.16) were the most stable

167   genes in 'Al treatment'.

168   To determine the optimal number of reference genes required for accurate normalization,

169   geNorm was used to calculate the pairwise variation (Vn/Vn+1) between the sequential

170   normalization factors (NFs) (NFn and NFn+1). As suggested, a threshold value of 0.15 was

171   adopted. As depicted in Fig. 2, pairwise variation analysis indicated that the ideal number of

172   reference genes may be different for the different groups. For instance, only two genes are

173   necessary for normalization for 'immature seed stage', 'abiotic cotyledons' and 'Al treatment',

174   but the pairwise variation of the other three groups was above the threshold of 0.15. The pairwise

175   variations for cotyledons and hypocotyls under different abiotic treatments are provided in

176   Supplemental Fig. S3.

177   Unlike geNorm, normFinder (Andersen et al. 2004) depends on a variance estimation

178   approach, which allows the comparison of inter/intra-group variation. Genes with the lowest

179   average expression stability values are the most stable. In the normFinder analysis, *FtH3* and

180   *FtCACS* were the most stable genes in 'abiotic cotyledons', and *FtSAND* and *FtExpressed1* were

181   the most stable genes under 'Al treatment'. In addition, the most stable reference genes in

182   'immature seed stage' were *FtSAND* and *FtGAPDH*, while the other three groups had the same

183   two most stable reference genes (*FtSAND* and *FtCACS*). The least stable gene in all groups was

184   *FtActin*.

185   BestKeeper (Pfaffl et al. 2004) determines the most stable genes by taking the coefficient of

186   variance (CV) and SD of the Cq values. The more stably expressed genes are indicated by the

187   lower SD and CV values. The results showed that the most stably expressed genes were *FtSAND*

188   (CV ± SD = 1.32 ± 0.33) and *FtCACS* (CV ± SD = 1.47 ± 0.31) for 'abiotic cotyledons'. In

189   'abiotic hypocotyls', *FtH3* (CV ± SD = 2.47 ± 0.51) and *FtSAND* (CV ± SD = 2.68± 0.69) were

190   the most stable genes. *FtH3* (CV ± SD = 2.55 ± 0.52, CV ± SD = 2.69 ± 0.54) and *FtCACS* (CV

191   ± SD = 2.70± 0.61, CV ± SD = 2.33± 0.53) were the most stable genes in the 'abiotic total' and

192   'total' groups, respectively. *FtExpressed1* (CV ± SD = 073 ± 0.17) and *FtEF-1α* (CV ± SD =

193   1.55 ± 0.25) showed the most stable expression in 'Al treatment', and *FtExpressed1* (CV ± SD =

194   2.00± 0.50) and *FtCACS* (CV ± SD = 2.43 ± 0.57) showed the highest expression stabilities in

195   'immature seed stage'. The least stable gene was *FtActin*, but the *FtGAPDH* gene, with a CV ±

196   SD of 7.59 ± 1.39 in 'abiotic hypocotyls', was considered the least acceptable for gene

197   expression normalization.

198   **Reference gene validation**

199   To validate the availability of a reference gene, the expression levels of *FtSTAR* under Al

200   treatment and of the *FtDFR* gene under UV treatment were determined using the seven candidate

201   reference genes for normalization. For Al treatment, *STAR* has a conserved response in plants,

202   and this has been shown in previous reports, such as reports on rice *STAR2* (Huang et al. 2009),

203   *Arabidopsis ALS* (Larsen et al. 2005) and tartary buckwheat *FtSTAR2* (Zhu et al. 2015), whose

204   expression increased after exposure to Al. The expression of *FtSTAR2* was greatly increased

205   (Fig. 3), which was reinforced by the Al-induced expression of *STAR2*. The use of the most

206   favourable reference gene (*FtExpressed1*) resulted in the greatest variation, resulting in increases

207   of approximately 1.67-fold in root tips and 6.24-fold in basal roots, and the use of the other most

208   stable gene (*FtEF-1a*) resulted in increases of approximately 1.42-fold and 6.19-fold,

209   respectively. Finally, the use of the least stable gene (*FtActin)* resulted in increases of

210   approximately 1.90-fold in root tips and 2.48-fold in basal roots. These genes also showed a

211   pattern of increased expression, regardless of whether stable or unstable reference genes were

212   used for normalization. However, the relative expression using the most stable genes showed the

213   higher fold increases compared with the unstable genes. Therefore, *FtExpressed1* and *FtEF-1a*
214   are the most suitable genes under Al treatment.
215   DFR catalyses the reduction of dihydroflavonols to leuco-anthocyanins and is a key enzyme in
216   the biosynthesis of anthocyanins (Yuan et al. 2007). The contents of anthocyanin increased under
217   UV treatment in tartary buckwheat hypocotyls (Eguchi & Sato 2009). In hypocotyls under UV
218   treatment, the relative quantification of *FtDFR* using the most stable genes (*FtCACS, FtH3* and
219   *FtActin)* for normalization exhibited similar expression patterns but different expression levels.
220   When other less stable genes were used for normalization, the results obviously differed from
221   those with the most stable genes (Fig. 4). The expression levels of the *FtDFR* gene under UV
222   treatment showed similar trends after normalization to stable genes in hypocotyls. Thus, we
223   selected the most stable reference genes for gene expression normalization in tartary buckwheat.
224

Discussion

226   SYBR Green I is a fluorescent reporter dye used in qRT-PCR; when it binds double-stranded
227   DNA, its fluorescence increases nearly 1000-fold (Morrison et al. 1998). The problems in qRT-
228   PCR caused by the variability of RNA templates and inappropriate data normalization are
229   obvious and widely known but disregarded (Nolan et al. 2006). To address these questions,
230   proper reference genes should be used. Thus, validating candidate reference genes and selecting
231   stable reference genes are becoming general interests for researchers.
232   Candidate reference genes can be selected from reference gene validation papers that use the
233   same experimental conditions or closely related species (orthologies) or by mining
234   transcriptomic data for stably expressed genes or traditional housekeeping genes (Hruz et al.
235   2011). The seven candidate reference genes tested in tartary buckwheat include three genes that
236   are stably expressed in common buckwheat (*SAND, CACS* and *Expressed1*), three commonly
237   used reference genes (*GAPDH, Actin* and *EF-1a)* and one unique gene (*H3*). Neither high (>30)
238   nor low (<15) Cq values are recommended based on general guidelines (Wan et al. 2010). In this
239   study, the Cq values of the reference genes ranged from 14.98 (*FtGAPDH*) to 29.65 (*FtActin*),
240   suggesting variable expression. Although the raw Cq value comparison can provide a rough
241   estimate of the stability of gene expression, it is not sufficient to accurately evaluate the
242   expression patterns of reference genes.

243   Scientists have developed several approaches to select stable reference genes for
244   normalization. However, to date, there is no consistent algorithm that can be used to test gene
245   stability. These software programs have their own merits and drawbacks: NormFinder can avoid
246   the misinterpretations caused by the artificial selection of co-regulated genes (Andersen et al.
247   2004); geNorm is based on the assumption that none of the analysed genes are co-regulated
248   (Vandesompele et al. 2002); and geNorm can estimate the fewest number of reference genes
249   needed for accurate normalization (Stamova et al. 2009). It seems possible that more reliable
250   controls can be obtained using a combination of different algorithms (de Almeida et al. 2010).
251   We used three software programs that were based on different statistical approaches to assess
252   seven candidate genes in tartary buckwheat. The distinct statistical algorithms are likely to
253   generate inconsistent rankings of stability. After integrating the outcomes of the three programs
254   above in this study, we recommend the reference genes in tartary buckwheat as follows: *FtCACS*
255   *+ FtSAND* for 'abiotic cotyledons'; *FtExpressed1 + FtEF-1α* for 'Al treatment'; *FtCACS +*
256   *FtExpressed1* for 'immature seed stage'; *FtCACS* for 'abiotic total'; and *FtH3* for 'abiotic
257   hypocotyls' and 'total'. The *H3* gene is the most commonly used internal gene for normalization
258   in tartary buckwheat (Luo et al. 2016). Our results indicate that *FtH3* is a stable gene under
259   abiotic treatment. However, it is not stable across different organs or after Al treatment. In
260   common buckwheat (Demidenko et al. 2011), *CACS* and *Expressed1* were validated as the most
261   stable genes in the development and fruit stages. *FtCACS* and *FtExpressed1* being the most
262   stable reference genes in the immature seed stage of tartary buckwheat supports the statement
263   that the orthologues of identified reference genes could serve the same purpose in other species.
264   In tomato, similar results were obtained in a study on reference gene selection (Exposito-
265   Rodriguez et al. 2008).

266   *GAPDH* and *Actin* are the most generally used reference genes for the analysis of gene
267   expression in various plant species (Kumar et al. 2011). However, our analysis indicates that
268   *GAPDH* and *Actin* are not reliable genes for comparative expression analysis. The cause of these
269   fluctuations at the gene expression level is probably that GAPDH and Actin have several
270   biological functions, such as participating in the glycolytic pathway and other processes
271   (Stürzenbaum & Kille 2001). Actin supports the cell and determines its shape, and it also takes
272   part in other cellular functions (Kravets et al. 2017). Although *Actin* is a stable reference gene in
273   developmental studies, it is not stable under various conditions.

Conclusions

274

275   As far as we know, our studies take advantage of three software (geNorm, NormFinder and

276   BestKeeper) to analysis the stability of seven candidate reference genes for the first time in

277   tartary buckwheat using qRT-PCR. Three software identified sightly differet genes as most

278   suited for normalization prompted us to merge the data. The result showed that the expression of

279   *GAPDH* or *Actin* is unstable across all samples. We also provide a list with the stable reference

280   genes in six group and under certain conditions.

281

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Figure 1

Cq values of seven candidate reference genes across all experimental sets in tartary buckwheat.

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

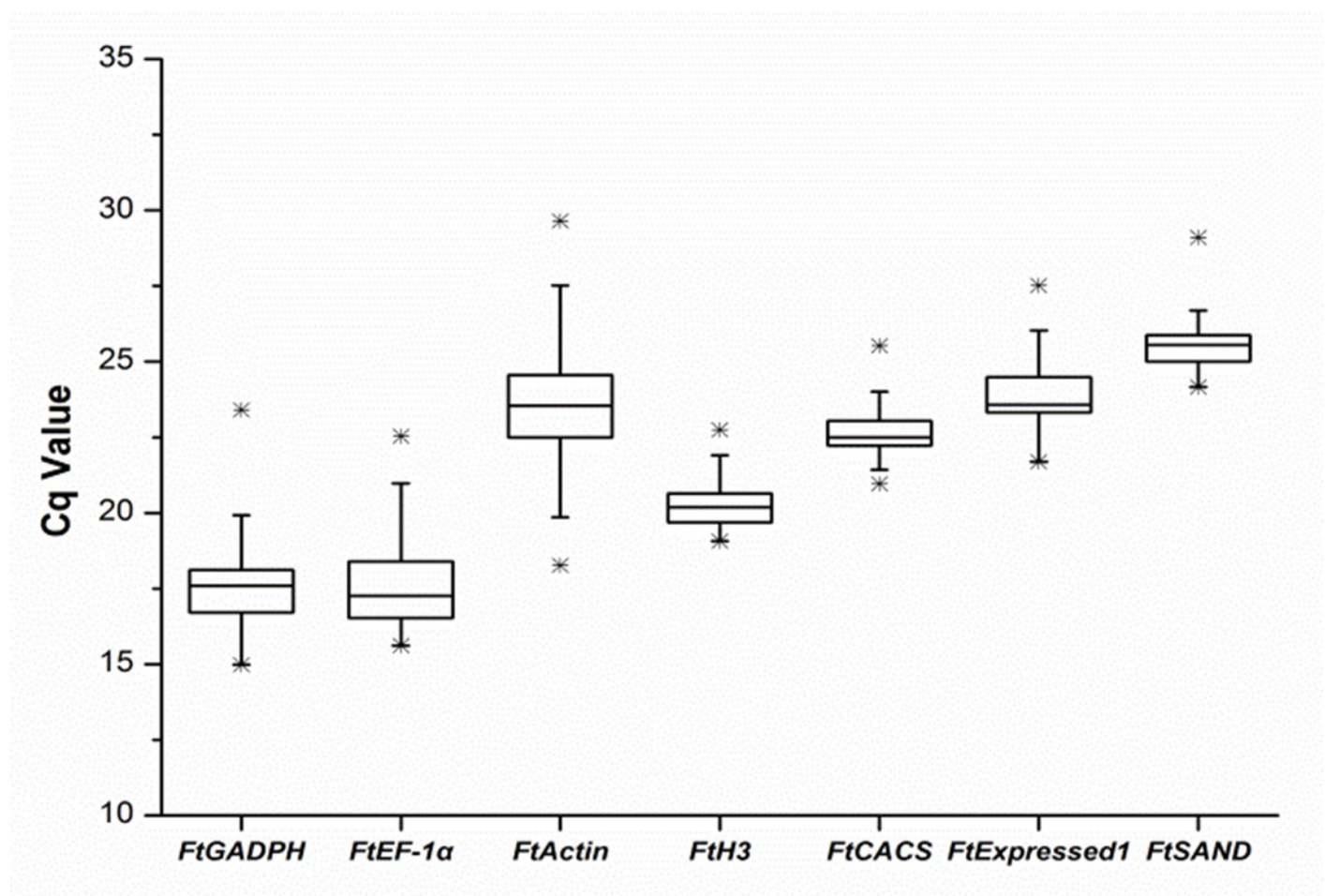



Figure 2

Optimal number of reference genes required for accurate normalization entile.

	'abiotic Hypocotyls'	'abiotic cotyledons'	'abiotic total'	'Al treatment'	'immature seed stage'	'total'
V2/3	0.228	0.14	0.191	0.072	0.125	0.208
V3/4	0.218	0.154	0.19	0.057	0.099	0.167
V4/5	0.176	0.196	0.215	0.06	0.113	0.205
V5/6	0.199	0.158	0.181	0.057	0.153	0.168
V6/7	0.19	0.348	0.277	0.135	0.276	0.308

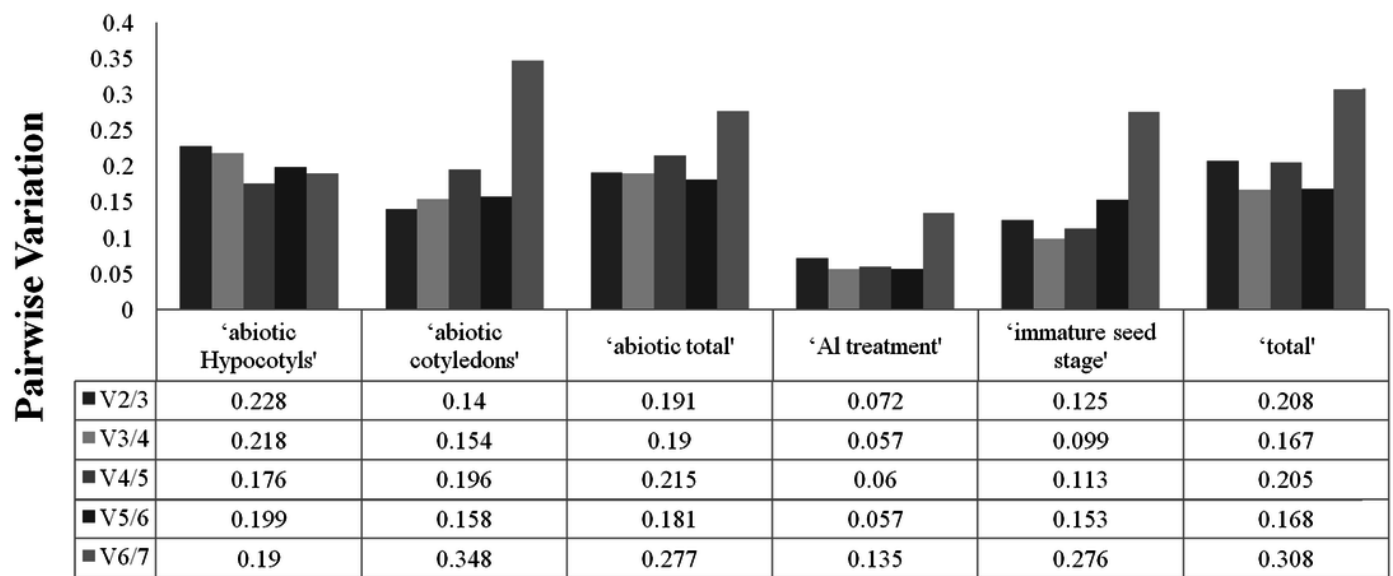



Figure 3

Relative quantification of *FtSTAR2* gene expression under Al treatment in tartary buckwheat.

Error bars represent standard deviation of the mean, data shown are means ± SD (n = 3).

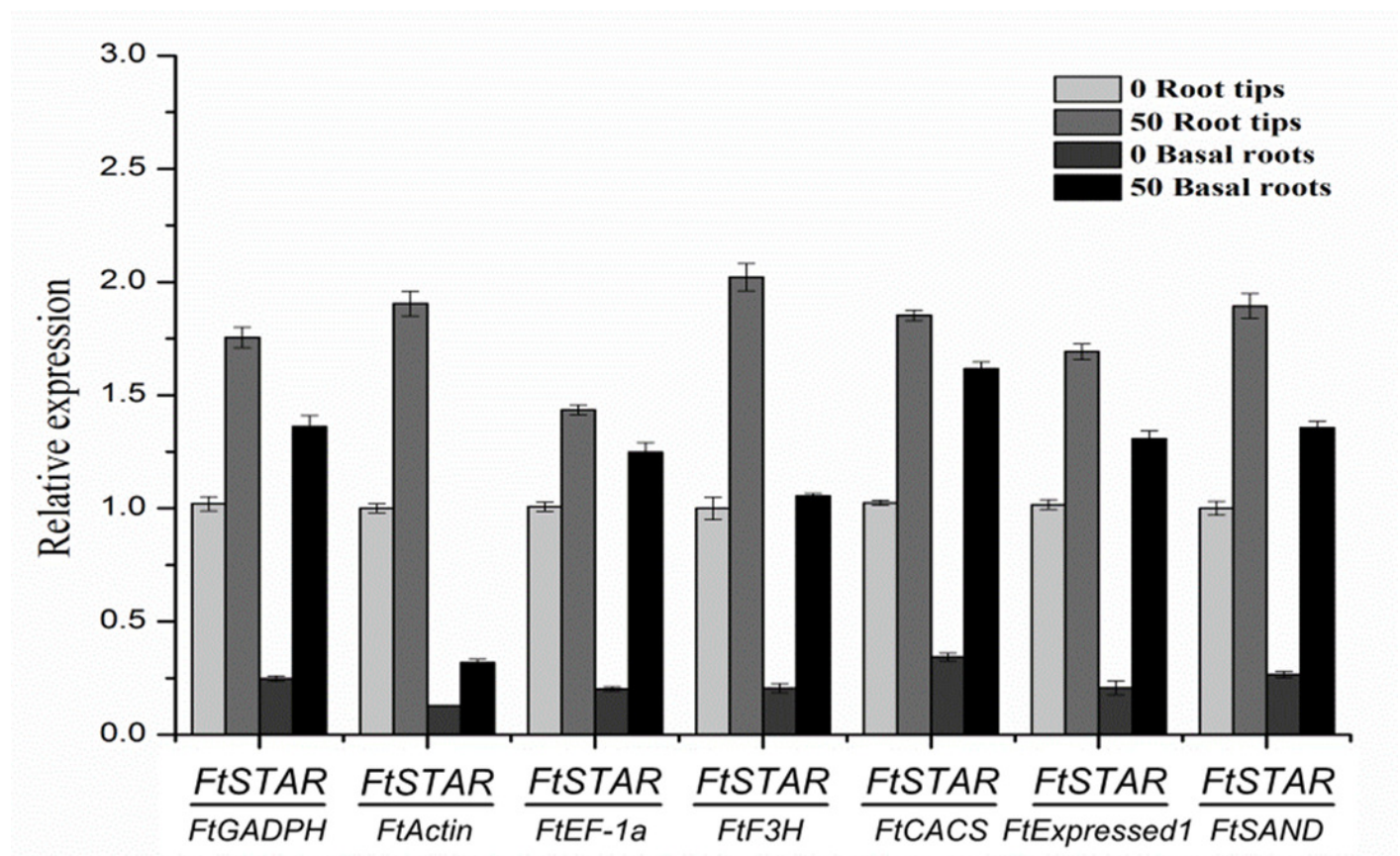
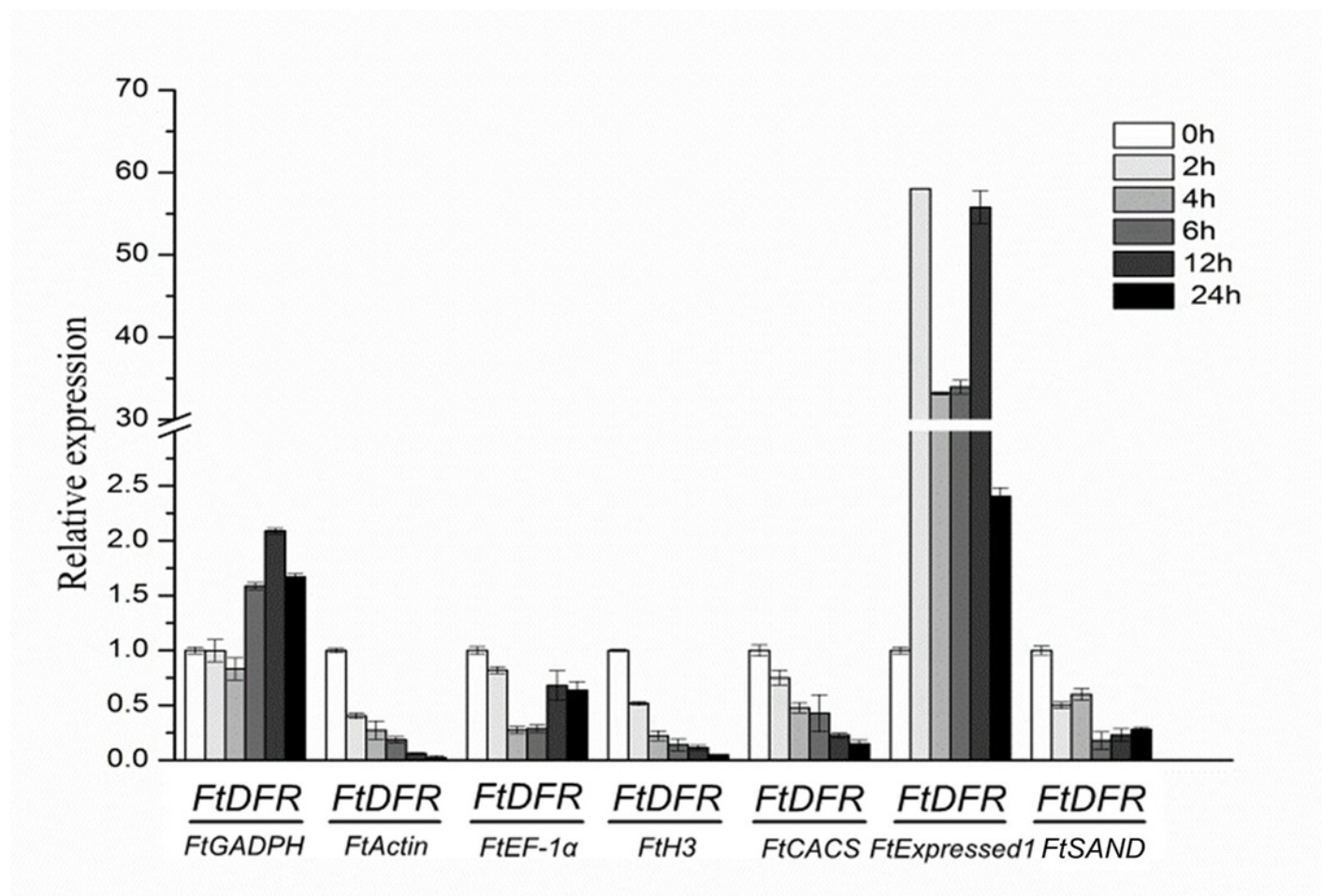



Figure 4

Relative quantification of *FtDFR* gene expression under UV treatment in tartary buckwheat hypocotyls.

Error bars represent standard deviation of the mean, data shown are means ± SD (n = 3).

Genes, primers and different features derived from qRT-PCR analysis

1   **Table 1** Genes, primers and different features derived from qRT-PCR analysis

Gene symbol	Gene name	Amplification length (bp)	Primer sequences	E%	TM℃
FtSAND	SAND family protein gene	79	GACCCCCTTGCAGACAAAGCATTGGCA TCTCGTTCTCAACGTCTTTTACCCACTGG	98.9	81
FtCACS	Clathrin adapter complex subunit family protein	125	AAGACAGTCAGTTTCGTGCCACCTGA TCCATGCGTGTTCTACCCAACTCCTT	90.3	82.5
FtExpressed1	Expressed protein of unknown function	127	AGGCCAGTTCCTGCTGAATGTAATGC TAGCCTGATCCAAACAAGCCTGGCAA	90.9	83
FtH₃	Histones 3	158	GAAATTCGCAAGTACCAGAAGAG CCAACAAGGTATGCCTCAGC	109.3	85
FtGAPDH	Glyceradehyde-3-phosphate debydrogenase gene	155	TGGAGCTGCTAAGGCTGTCG TGATAGCACTCTTGATGTCCTCGTA	91.9	83
FtActin	Actin 2 genes	118	GGAAGTATAGCGTCTGGATTGGC CACTTGCGGTGAACGATTGC	93.1	82.5
FtEF-1α	Elongation factor-1α gene	108	GCTGCTGAGATGAACAAGAGGTC CTCAAACTTCCACAACGCGAT	91.2	82.5

The stability of the expression of seven candidate reference genes in six groups as analysed by geNorm, NormFinder, BestKeeper

1 **Table 2** The stability of the expression of seven candidate reference genes in six groups as

2 analysed by geNorm, NormFinder, BestKeeper

Group	Rank	geNorm		NormFinder		BestKeeper		
		Gene	Stability	Gene	Stability	Gene	SD	CV
Abiotic Cotyledons	1	FtCACS	0.33	FtH₃	0.058	FtSAND	0.33	1.32
	2	FtH₃	0.33	FtCACS	0.080	FtCACS	0.31	1.47
	3	FtSAND	0.41	FtSAND	0.129	FtH₃	0.33	1.64
	4	FtExpressed1	0.54	FtExpressed1	0.280	FtExpressed1	0.59	2.48
	5	FtEF-1α	0.73	FtEF-1α	0.683	FtEF-1α	1.04	5.70
	6	FtGAPDH	0.84	FtGAPDH	0.751	FtGAPDH	1.07	6.26
	7	FtActin	1.31	FtActin	1.678	FtActin	1.46	6.36
Abiotic Hypocotyls	1	FtH₃	0.65	FtSAND	0.416	FtH₃	0.51	2.47
	2	FtSAND	0.65	FtCACS	0.437	FtSAND	0.69	2.68
	3	FtCACS	0.72	FtH₃	0.449	FtActin	0.79	3.38
	4	FtExpressed1	0.84	FtEF-1α	0.469	FtExpressed1	0.81	3.34
	5	FtEF-1α	0.91	FtExpressed1	0.475	FtCACS	0.86	3.75
	6	FtGAPDH	1.05	FtGAPDH	0.811	FtEF-1α	0.92	5.32
	7	FtActin	1.17	FtActin	0.895	FtGAPDH	1.35	7.59
Abiotic total	1	FtH₃	0.55	FtCACS	0.304	FtH₃	0.52	2.55
	2	FtSAND	0.55	FtSAND	0.327	FtCACS	0.61	2.70
	3	FtCACS	0.61	FtH₃	0.349	FtSAND	0.63	2.46
	4	FtExpressed1	0.72	FtExpressed1	0.380	FtExpressed1	0.73	3.02
	5	FtEF-1α	0.89	FtEF-1α	0.694	FtEF-1α	1.02	5.71
	6	FtGAPDH	1.01	FtGAPDH	0.782	FtActin	1.17	5.07
	7	FtActin	1.30	FtActin	1.329	FtGAPDH	1.20	6.89
Al treatment	1	FtEF-1α	0.16	FtSAND	0.091	FtExpressed1	0.17	0.73
	2	FtExpressed1	0.16	FtExpressed1	0.100	FtEF-1α	0.25	1.55
	3	FtSAND	0.21	FtH₃	0.105	FtH₃	0.31	1.56
	4	FtGAPDH	0.23	FtEF-1α	0.106	FtGAPDH	0.34	1.88
	5	FtH₃	0.27	FtGAPDH	0.156	FtCACS	0.35	1.57
	6	FtCACS	0.30	FtCACS	0.282	FtSAND	0.37	1.43
	7	FtActin	0.49	FtActin	0.652	FtActin	1.05	4.16
Immatur	1	FtExpressed1	0.16	FtSAND	0.097	FtExpressed1	0.50	2.00

e seed stage	2	*FtCACS*	0.16	*FtGAPDH*	0.097	*FtCACS*	0.57	2.43
	3	*FtSAND*	0.30	*FtH₃*	0.167	*FtSAND*	0.82	3.03
	4	*FtGAPDH*	0.36	*FtCACS*	0.292	*FtEF-1α*	0.83	4.32
	5	*FtH₃*	0.45	*FtExpressed1*	0.422	*FtGAPDH*	0.86	4.51
	6	*FtEF-1α*	0.62	*FtEF-1α*	0.675	*FtH₃*	1.11	5.29
	7	*FtActin*	1.01	*FtActin*	1.354	*FtActin*	2.16	9.96
Total	1	*FtH₃*	0.60	*FtCACS*	0.245	*FtCACS*	0.53	2.33
	2	*FtCACS*	0.60	*FtSAND*	0.304	*FtH₃*	0.54	2.69
	3	*FtSAND*	0.66	*FtH₃*	0.313	*FtSAND*	0.57	2.24
	4	*FtExpressed1*	0.71	*FtExpressed1*	0.377	*FtExpressed1*	0.68	2.85
	5	*FtGAPDH*	0.87	*FtGAPDH*	0.650	*FtGAPDH*	0.97	5.53
	6	*FtEF-1α*	0.97	*FtEF-1α*	0.780	*FtEF-1α*	1.03	5.91
	7	*FtActin*	1.33	*FtActin*	1.480	*FtActin*	1.36	5.70