Evaluation of putative reference genes for quantitative real-time PCR normalization in *Lilium regale* during development and under stress

Qiang Liu, Chi Wei, Ming-Fang Zhang, Gui-Xia Jia

Normalization to reference genes is the most common method to avoid bias in real-time quantitative PCR (qPCR), which has been widely used for quantification of gene expression. Despite several studies on gene expression, *Lilium*, and particularly *L. regale*, has not been fully investigated regarding the evaluation of reference genes suitable for normalization. In this study, nine putative reference genes, namely *18S rRNA*, *ACT*, *BHLH*, *CLA*, *CYP*, *EF1*, *GAPDH*, *SAND* and *TIP41*, were analyzed for accurate quantitative PCR normalization at different developmental stages and under different stress conditions, including biotic (*Botrytis elliptica*), drought, salinity, cold and heat stress. All these genes showed a wide variation in their Cq (quantification cycle) values, and their stabilities were calculated by geNorm, NormFinder and BestKeeper. In a combination of the results from the three algorithms, *BHLH* was superior to the other candidates when all the experimental treatments were analyzed together; *CLA* and *EF1* were also recommended by two of the three algorithms. As for specific conditions, *EF1* under various developmental stages, *SAND* under biotic stress, *CYP/GAPDH* under drought stress, and *TIP41* under salinity stress were generally considered suitable. All the algorithms agreed on the stability of *SAND* and *GAPDH* under cold stress, while only *CYP* was selected under heat stress by all of them. Additionally, the selection of optimal reference genes under biotic stress was further verified by analyzing the expression level of *LrLOX* in leaves inoculated with *B. elliptica*. Our study would be beneficial for future studies on gene expression and molecular breeding of *Lilium*.

1 **Evaluation of putative reference genes for quantitative real-**

2 **time PCR normalization in *Lilium regale* during development**

3 **and under stress**

4 Qiang Liu[1*], Chi Wei[1*], Ming-Fang Zhang[1], Gui-Xia Jia[1]

5 [1] Beijing Key Laboratory of Ornamental Plants Germplasm Innovation & Molecular Breeding, National

6 Engineering Research Center for Floriculture, Beijing Laboratory of Urban and Rural Ecological

7 Environment and College of Landscape Architecture, Beijing Forestry University, Beijing, PR China

8 * These authors contributed equally to this work.

10 Corresponding Author:

11 Gui-Xia Jia [1]

12 Tsinghua East Road No.35, Haidian District, Beijing 100083, PR China

13 Email address: gxjia@bjfu.edu.cn

15 **Abstract:** Normalization to reference genes is the most common method to avoid bias in real-time

16 quantitative PCR (qPCR), which has been widely used for quantification of gene expression.

17 Despite several studies on gene expression, *Lilium*, and particularly *L. regale*, has not been fully

18 investigated regarding the evaluation of reference genes suitable for normalization. In this study,

19 nine putative reference genes, namely *18S rRNA*, *ACT*, *BHLH*, *CLA*, *CYP*, *EF1*, *GAPDH*, *SAND*

20 and *TIP41*, were analyzed for accurate quantitative PCR normalization at different developmental

21 stages and under different stress conditions, including biotic (*Botrytis elliptica*), drought, salinity,

22 cold and heat stress. All these genes showed a wide variation in their Cq (quantification cycle)

23 values, and their stabilities were calculated by geNorm, NormFinder and BestKeeper. In a

24 combination of the results from the three algorithms, *BHLH* was superior to the other candidates

25 when all the experimental treatments were analyzed together; *CLA* and *EF1* were also

26 recommended by two of the three algorithms. As for specific conditions, *EF1* under various

27 developmental stages, *SAND* under biotic stress, *CYP/GAPDH* under drought stress, and *TIP41*

28 under salinity stress were generally considered suitable. All the algorithms agreed on the stability

29 of *SAND* and *GAPDH* under cold stress, while only *CYP* was selected under heat stress by all of

30 them. Additionally, the selection of optimal reference genes under biotic stress was further verified

31 by analyzing the expression level of *LrLOX* in leaves inoculated with *B. elliptica*. Our study would

32 be beneficial for future studies on gene expression and molecular breeding of *Lilium*.

33 **Keywords:** Reference genes; Normalization; *Lilium regale*; Development stages; Biotic Stress;

34 Abiotic Stress.

Introduction

37   As one of the standard methods for gene expression profiling, real-time quantitative PCR

38 (qPCR) is featured with a high sensitivity, specificity and throughput in comparison with other

39 previous molecular techniques (Gachon et al. 2004). During the qPCR assay, data normalization

40 is a prerequisite for accurate and reproducible measurement of quantitative expression. A common

41 strategy for normalization is to normalize the mRNA expression of the gene of interest to a

42 reference gene. Accordingly, the accuracy of the measurement is directly relied on the expression

43 stability of the reference gene in different samples, which may vary in tissues or organs,

44 developmental stages or the experimental conditions (Radonić et al. 2004). A number of

45 housekeeping genes are commonly used as reference genes, such as *glyceraldehyde-3-phosphate*

46 *dehydrogenase* (*GAPDH*), *18S ribosomal RNA* (*18S rRNA*), *Elongation factor 1-*α (*EF1*) and *Actin*

47 (*ACT*) (Czechowski et al. 2005; Gutierrez et al. 2008). These genes are involved in basic and

48 ubiquitous cellular processes including glycolytic metabolism, ribosome biosynthesis,

49 cytoskeleton constitution and protein folding. It is assumed that such genes are expressed at a

50 constant level in all tissues independently of growing environment (Dheda et al. 2004). However,

51 it is evidenced that the transcription levels of these housekeeping genes can be unstable in response

52 to different experimental conditions or in different tissue types, leading to errors in quantification

53 of target genes (Nicot 2005; Schönbach et al. 2012). Therefore, it is necessary to verify the

54 expression stability of candidate reference genes across various experimental treatments or tissue

55 types (Bin et al. 2012; Nicot 2005). Several statistical algorithms, including geNorm

56 (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al.

57 2004), were introduced to identify the most suitable gene among a set of candidates for qPCR

58 normalization. Based on these algorithms, evaluation of putative reference genes has been

59 performed on a number of plant species (Bin et al. 2012; Fu et al. 2012; Nicot 2005; Schönbach et

60 al. 2012).

61   As a bulbous flowering plant with great ornamental value, *Lilium* is one of the most important

62 floricultural crops in the world. Thousands of cultivars in this genus were produced under inter-

63 specific hybridization and artificial selection in the last twenty years. However, only a limited

64 number of studies have been carried out on the gene expression of *Lilium,* and the reference genes

65 used in these studies, namely *ACT*, *18S rRNA* or *GAPDH* (He et al. 2014; Li et al. 2014; Tong et

66 al. 2013; Wang et al. 2009; Xin et al. 2010), was only based on the results from other species

67 without an experimental comparison among the candidate reference genes of *Lilium*. Only until

68 very recently the evaluation of reference genes in *L. davidii* var. *unicolor* and *L. brownii* has been

69 reported (Li et al. 2015; Luo et al. 2014), but specific studies on these genes under certain

70 conditions, such as different developmental stages or stresses, are still needed for more accurate

71 normalization in qPCR. As a promising material for breeding and gene study in section Leucolirion

72 of *Lilium*, *L. regale* is a wild lily species distributing across Min River basin in Sichuan Province

73 of China. It was found to possess extremely high resistance to biotic and abiotic stresses (Zhao et

74 al. 1994). Studies on its reference genes under environmental stresses would be essential for

75 analyzing its expression pattern and understanding the mechanism of stress resistance, and

76 providing guidance to the molecular breeding of lilies.

77   Because the knowledge on gene expression profiling in *Lilium* is still limited, transcriptome

78 and digital gene expression (DGE) technology would play a pivotal role in the reference gene

79 selection. Therefore, in this study, the expression stability of nine putative reference genes were

80 evaluated under different developmental stages and various stress treatments. Three statistical

81 algorithms, geNorm, NormFinder and BestKeeper, were combined to analyze the expression

82 stabilities of these candidate reference genes.

83 ## Materials and Methods

84 **Plant materials and sampling**

85   Bulbs of *L. regale* with a circumference of 120 mm were stored at 4 °C for 3 months, and

86 then planted in pots (diameter 100 mm) filled with substrate (sterile turf: vermiculite: perlite = 1:

87 1: 1, v/v/v). They were grown in a growth chamber with 12 h light (200 μmol·m^{-2}·s^{-1})/12 h dark

88 photoperiod and 25 °C day/22 °C night thermoperiod, at a relative humidity of 80%. The plants

89 were daily watered to maintain the field capacity to the maximum. For quantification at different

90 developmental stages, one leaf of each plant was sampled every month after germination. The

91 sampling was conducted for six months from the fifth to tenth leaf below the apex, throughout the

92 vegetative and reproductive stages of the plants before they entered senescence.

93   The plants generated from the bulbs were used for other treatments, which were conducted

94   during the flower bud stage. For drought stress treatment, the water supply was withheld and leaf

95   samples were collected at 2, 3, 4, 6 and 8 d during the treatment. For salinity stress treatment, the

96   pots with plants were completely saturated with water containing 100 mM NaCl, followed by leaf

97   sampling at 2, 6, 12, 24, 36 and 48 h during the treatment. For heat or cold stress treatment, the

98   pots with plants were transferred to another growth chamber with a same photoperiod at 37 °C

99   (heat stress) or 4 °C (cold stress). Leaves from the treated plants were collected at 2, 6, 12, 24, 36

100   and 48 h during the treatment. One leaf of each plant was sampled at every time point from the

101   fifth to tenth leaf below the apex.

102   For biotic stress treatment, the fifth to tenth leaf below the apex of each plant were detached

103   and surface-sterilized by 0.1% sodium hypochlorite for inoculation. *Botrytis elliptica* was cultured

104   on potato dextrose agar medium (PDA) under near-UV light for 5 days. The conidia were collected

105   by successive gentle vortexing in Tween 20 solution (0.05% Tween 20 in sterile deionized water).

106   After adjusted to 5×10^4 conidia·mL^{-1}, the suspension was sprayed on the abaxial surface of the

107   leaves. Mock inoculations with water were also performed as a control group. The petioles of

108   inoculated leaves were covered with cotton infiltrated by fertilized water, and the leaves were

109   incubated on wet filter papers in petri dishes, which were placed in a growth chamber with 100%

110   relative humidity, 12 h light (200 μmol·m^{-2}·s^{-1})/12 h dark photoperiod and 25 °C day/22 °C night

111   thermoperiod. Samples were collected at 2, 6, 12, 24, 36 and 48 hpi (hours post inoculation).

112   All the samples collected were immediately frozen in liquid nitrogen and stored at -80 °C.

113   Two biological replications were designed for every developmental stage or every time point

114   during the stress treatments, each replication consisting of three leaves from three individual plants

115   as a sample pool. 12 sample pools from each treatment (except 10 from drought treatment), namely

116   70 sample pools from 210 plants in total were used for study.

117   **Total RNA extraction and cDNA synthesis**

118   Total RNA from each sample was extracted using the EASYspin Plus Plant RNA kit (RN38,

119 Aidlab Biotech, PR China) according to the manufacturer's protocol. Each RNA sample was

120 treated with an RNase-free DNase I (Promega, USA) at 37 °C for 30 min to eliminate the trace

121 contaminants of genomic DNA. The concentration and integrity of the samples were examined by

122 NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and 1.4% agarose gel

123 electrophoresis. The first strand cDNA was synthesized based on 1 µg total RNA of each sample

124 using the M-MLV reverse transcription system (Promega, USA) according to the manufacturer's

125 protocol.

126 **Primer design and PCR confirmation**

127 Primers were designed using Beacon designer 7 (Premier Biosoft, USA) following the

128 stringent criteria: PCR product size between 75–200 bp, primer length between 18–25 bp, optimal

129 T_m between 55–65 °C, GC content between 40%–60%. The theoretical T_m of the amplicon for

130 each gene were predicted by Primer Premier 5 (Premier Biosoft, USA), in which the salt

131 concentration was set as 50 mM. In order to check the specificity and suitable reaction condition

132 of the primers, they were initially tested in a general PCR reaction with gradient temperature (52–

133 60 °C) using cDNA as a template. For further confirmation, PCR products were cloned into

134 pGEM-T Easy vectors (Promega, USA) respectively and then sequenced by Sangon Biotech (PR

135 China).

136 **qPCR analysis and efficiency**

137 Real-time quantitative PCR was conducted with MiniOpticon Real-time PCR System (Bio-

138 Rad, USA), using SYBR Premix Ex Taq (Takara Bio, Japan) to monitor dsDNA synthesis. It was

139 carried out in a 20 µL mixture containing 2 µl cDNA template, 10 µl 2× SYBR Premix Ex Taq,

140 and each primer in the optimized concentration shown in Table 1, by the following thermal cycles:

141 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, the optimized annealing temperature

142 shown in Table 1 for 10 s and 72 °C for 15 s. In order to ensure the specificity of each primer pair,

143 melting curves were recorded after Cycle 40 by heating from 65 °C to 95 °C stepwise by 0.5 °C

144 every 5 s. Each qPCR reaction was performed in technical triplicate.

145   The reaction condition including the best primer concentration and annealing temperature

146   was optimized using the cDNA sample pool of the total samples used in this study as templates,

147   which were analyzed as five serial ten-fold dilutions. The amplification efficiency of each primer

148   pair was derived from a standard curve generated after the optimization. Mean quantification cycle

149   (Cq) values of each ten-fold dilution were plotted against the logarithm of the cDNA dilution factor

150   (Ginzinger 2002).

151   **Data analysis**

152   The Cq values for each tested reference gene were analyzed by a specific threshold. The raw

153   Cq values were converted into relative quantities using the formula $Q = E^{-\Delta Cq}$, where E is the

154   efficiency of the gene amplification for each primer pair and ΔCq is the lowest Cq value as

155   calibrator (which corresponds to the sample with the highest expression) minus the Cq value of

156   the sample tested. The data obtained for each primer were analyzed using geNorm V3.5

157   (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al.

158   2004) for reference gene selection.

159   **Normalization of *LrLOX***

160   The gene *LrLOX* (GenBank No. KM051414), a putative homolog of *lipoxygenases* (*LOXs*)

161   in *L. regale*, was used to validate the selected reference genes. The primers were designed using

162   Beacon designer 7 (Forward: 5′-TTCCAGCGACAACAGGAGCAC-3′; Reverse: 5′-

163   CGTCGTCCACCAAATCCACTT-3′). The leaves of *L. regale* were inoculated with *B. elliptica*

164   using the method mentioned above. The expression profiling of *LrLOX* in the samples collected

165   at 2, 6, 12, 24, 36 and 48 hpi was analyzed under the same experimental procedure as above. The

166   samples at 0 hpi were taken as control group. For comparative purposes, the relative expression of

167   the target gene was calculated with different normalization factors based on the geometric mean

168   of the three most stable genes and the most unstable genes under biotic stress, as well as the most

169   stable genes in all conditions concluded from the analysis. The relative expression was calculated

170   by standard $E^{-\Delta\Delta Cq}$ method. The data collected were subjected to one-way ANOVA and the means

171   were analyzed by Tukey's range test. Data were represented as mean ± SD.

Results

Selection of candidate reference genes and amplification specificity

174   In our previous study, a cDNA library of *L. regale* was constructed to analyze the gene

175   expression pattern after inoculation with *B. elliptica* (Unpublished). Based on the RPKM (Reads

176   per Kb per million reads) values of the unigenes in the library (Table S1), as well as the previous

177   studies on reference gene selection in other plants (Bin et al. 2012; Fu et al. 2012), a total of nine

178   genes associated with a wide variety of biological functions were selected as candidate reference

179   genes during development or under biotic and abiotic stress: *18S rRNA*, *ACT*, *BHLH*, *CLA*, *CYP*,

180   *EF1*, *GAPDH*, *SAND* and *TIP41*. Their full names, cellular functions and EST GenBank accession

181   numbers are listed in Table 1. From at least three primer pairs per gene designed, the final primer

182   pairs for the nine genes were selected on the basis of their single PCR products, amplification

183   efficiencies and regression coefficients (R^2), as shown in Table 2. The primers for each gene

184   amplified a single PCR product at an expected size as confirmed in a 2% agarose gel

185   electrophoresis (Fig. S1) and later sequencing (Seq. S1). Melting curve analysis also confirmed

186   the single PCR products with no primer-dimers (Fig. S2), and their melting temperatures were

187   consistent with the prediction (Table 2). Under optimized reaction conditions (Table 2), their R^2

188   values ranged from 0.992 to 0.999, and amplification efficiencies ranged from 93.0% to 105.4%.

Transcription profiling of the reference genes

190   The expression levels of the nine candidate reference genes were assessed for expression

191   stability under diverse conditions including different developmental stages and biotic or abiotic

192   stresses. The Cq (quantification cycle) values for the candidate reference genes in all the samples

193   were used to compare the expression levels among these genes (Fig. 1). The data analysis showed

194   that these reference genes were moderately abundant in all tested samples, exhibiting a wide range

195   of expression levels. The mean Cq values of the reference genes varied from 18.88 to 24.40, with

196   most lying between 20 and 23. *GAPDH* showed the highest expression level in all the samples

197   with the lowest Cq value (18.89), while *SAND* showed the lowest expression level with the highest

198   Cq value (24.41). *CYP, ACT* and *GAPDH* showed great variability with the Cq value ranging from

199   18.40 to 27.65, 19.90 to 26.85 and 16.37 to 23.29 respectively. Since the expression levels of the

200   nine candidate reference genes showed a variation across all samples, further evaluation would be

201   necessary to identify the best selection(s) from these candidate genes for normalizing gene

202   expression under certain experimental conditions.

203   **Gene expression stability analysis**

204   Expression stabilities of the nine candidate reference genes were analyzed by geNorm and

205   NormFinder. The geNorm program calculated average expression stability (M) for a reference

206   gene based on the average pairwise variation between all genes tested (Vandesompele et al. 2002).

207   The rankings of the nine candidate reference genes under different conditions according to the M

208   values are depicted in Fig. 2. When all the samples were taken together, *BHLH, SAND, CLA* and

209   *EF1* (in order, similarly hereinafter) were the most stably expressed genes, while *CYP* and *18S*

210   *rRNA* were the least stable ones. In leaves across various developmental stages, *EF1*, *SAND*, *TIP41*

211   and *GAPDH* performed well, while *CYP* and *BHLH* were the least stable genes. *CLA*, *SAND* and

212   *EF1* had the most stable expression under biotic stress, whereas *CYP* and *18S rRNA* were the most

213   variably expressed of the nine candidate genes. Under drought stress, *CYP*, *SAND*, *GAPDH* and

214   *TIP41* were the most stable genes. By contrast, the most stable ones for salinity stress were *CLA*,

215   *TIP41*, *BHLH* and *GAPDH*. In response to cold and heat stress, *GAPDH*, *CYP* and *SAND* were

216   identified as the best two reference genes for both treatments.

217   The geNorm program was also used to determine the optimal number of reference genes

218   required for the normalization across different sets of experiment by calculating pairwise variation

219   (V_n/V_{n+1}) value. According to the instruction, 0.15 was suggested to be the cut-off value for

220   determining the optimal number of reference genes, below which the inclusion of additional

221   reference genes is not required (Vandesompele et al. 2002). For various developmental stages, the

222   V4/5 value was 0.145 as shown in Fig. 3, which means four reference genes, namely *EF1*, *SAND*,

223   *TIP41* and *GAPDH* should be considered. The experimental data for biotic stress (V2/3 value =

224   0.098) showed that *CYP* and *SAND* were sufficient for normalization. For drought stress (V2/3

225   value = 0.088) and salinity stress (V2/3 value = 0.051), the suitable reference genes were

226   *CYP*/*SAND* and *CLA*/*TIP41*, respectively. In addition, the V2/3 value of cold stress was 0.059,

227   indicating that these two reference genes, *GAPDH* and *TIP41*, were sufficient for normalization of

228   gene expression. *EF1* and *GAPDH* produced a V2/3 value of 0.085 in heat stress treatment,

229   suggesting these two candidate genes could be selected as appropriate reference genes. However,

230   the V_n/V_{n+1} values were higher than 0.15 when taken all the samples together, and thus the cut-off

231   value of 0.15 was a little strict in this case (Fernandez et al. 2011). The V5/6 value was 0.158,

232   indicating that the optimal number of reference targets would be five: *BHLH, SAND, CLA, EF1*

233   and *TIP41*.

234   In general, similar results were obtained when the candidate reference genes were analyzed

235   through NormFinder, which is based on variance estimation to calculate the normalization factor

236   (NF) (Andersen et al. 2004). These genes were ranked according to their stabilities under a given

237   set of experimental treatments (Table 3). The ranking under biotic stress was similar as that of

238   geNorm analysis. *CYP* and *TIP41* were also recognized as the most stable genes under drought

239   and salinity stress, respectively, while differences were observed in the analysis of genes under

240   cold and heat stress, where *SAND* and *CYP* were ranked as the most stable one respectively instead.

241   *TIP41* and *ACT* were the most stable genes for various developmental stages. When all the samples

242   were considered, *TIP41, EF1, BHLH* and *SAND* were the most stably expressed genes suggested

243   by NormFinder, while *CYP* and *18S rRNA* were suggested as the least stable genes by both the

244   algorithms.

245   Their expression stability of expression was re-analyzed using the BestKeeper algorithm,

246   which provides the standard deviation (SD) based on the Cq values of all candidate reference

247   genes, as well as the correlation coefficients (r) of the genes with the BestKeeper Index calculated

248   from the geometric mean of the remaining reference genes (Pfaffl et al. 2004). As shown in Table

249   4, the genes with a SD [± CP] value below 1 and a SD [± x-fold] value below 2 are considered to

250   be qualified for the gene expression normalization, and their r values (correlation coefficient) are

251   used for ranking. According to these criteria, *EF1*, *BHLH* and *CLA* were suggested as the three

252   most stable genes when all the experimental conditions were taken into consideration. During

253   different developmental stages, *EF1*, *SAND* and *CLA* were selected by the program for the most

254   stable normalization. Almost all the reference genes were within acceptable criteria under various

255   stresses, and the results were partially similar with those obtained by NormFinder.

256   Because of the diverse suggestions made by the three algorithms regarding the most stable

257   reference genes, we used Venn charts to identify the most suitable ones under certain conditions

258   by combining the top three genes for each algorithm together (Fig. 4). All the algorithms agreed

259   that *BHLH* was the most stably expressed gene across all the experimental conditions, while *CLA*

260   and *EF1* were recommended by two of the three programs respectively. As a synthesis of the three

261   programs' output, *EF1* under various developmental stages, *SAND* under biotic stress,

262   *CYP/GAPDH* under drought stress, and *TIP41* under salinity stress were generally considered

263   suitable. All the three algorithms were consistently in agreement on the stability of *SAND* and

264   *GAPDH* under cold stress, while only *CYP* was selected by all the three programs as for heat stress.

265   Notably, *ACT* and *18S rRNA*, the most commonly used reference genes, were hardly found to be

266   among the most stable transcripts as suggested by our results.

267   **Normalization of *LrLOX***

268   The expression level of *LrLOX* after inoculation with *B. elliptica* was calculated with the

269   reference genes suggested as above. According to the result of the evaluation for biotic stress,

270   *SAND* was the most stable reference genes, while *CYP* was the least stable one among the nine

271   candidate genes. *ACT* and *CLA* were also recommended by two of the three algorithms, but *ACT*

272   was considered to have a relatively lower stability than *CLA* and *SAND*. As shown in Fig. 5, when

273   normalized with *SAND*, the transcription level of *LrLOX* was down-regulated (fold change (FC) =

274   0.65) at 2 hpi, and a maximum level (FC = 2.23) was obtained at 12 hpi (hours post inoculation)

275 relative to uninoculated controls. Upregulation was still observed at 24 hpi, but the mRNA level

276 was then steadily decreased in the next 24 h. Similar expression patterns were generated when

277 normalized with *CLA* separately or in combination with *SAND* together. When normalized using

278 *ACT*, the expression level of *LrLOX* at 24 hpi was shown as down-regulated (FC = 0.87), but it

279 showed no significant difference if compared with the value normalized with *SAND* (Fig. 5).

280 Normalization with a combination of the three most stable genes also made a consistent result.

281 When normalized with *BHLH*, which was considered as the most stable gene overall but was

282 ranked in the middle under biotic stress, the expression pattern of *LrLOX* was still roughly

283 recognizable. However, significant difference could be observed at 12 hpi, as the maximum

284 mRNA level was reduced to 1.70 fold. As expected, the expression profile showed more variation

285 when normalized with *CYP*, the least stable genes calculated by the geNorm. Apart from the

286 incorrect down-regulation at 24 hpi here, the upregulation at 12 hpi was even as high as 6.66 fold.

287 **Discussion**

288 The accuracy of normalization in qPCR is directly relied on the expression stability of the

289 reference genes (Gachon et al. 2004). Thus, the selection of stable reference gene for a wide range

290 of conditions is crucial for quantification of gene expression. In our study, three popular statistical

291 algorithms, BestKeeper, geNorm and NormFinder, were used for evaluation of stable reference

292 genes in *L. regale*. NormFinder estimates both the intra- and inter-group variation and combines

293 them into a stability value (Andersen et al. 2004), and GeNorm evaluates the stability of reference

294 gene by pairwise comparison of expression ratio variation of among reference genes in specific

295 experimental sets (Vandesompele et al. 2002). These two algorithms both correct for inter-sample

296 variation, but BestKeeper do not account for differences in RNA quality/input or reverse

297 transcription (RT) efficiency across samples (Jiang et al. 2014). According to the results, the

298 suitable genes under each condition selected by these algorithms share much in common.

299 Moreover, geNorm and NormFinder were also in agreement on the least stable gene for each

300 treatment. The accordance in the results provided by these algorithms was also mentioned in some

301   previous study on other plants (De Spiegelaere et al. 2015; Mafra et al. 2012). However, obvious

302   divergence in these results still exists in some situations, mainly caused by the varying priorities

303   in different algorithms. BestKeeper even provides multiple factors in its result, thus different sort

304   criteria would also affect the final ranking. Since every algorithm has its own risks and benefits

305   for the evaluation, it is generally accepted to combine these results together for optimal selection

306   of suitable reference genes (Fig. 4).

307   Several studies involving the gene expression in *Lilium* have been reported recently.

308   However, due to the absence of an extensive evaluation of the reference genes in *Lilium*, the

309   selection of them was only based on previous experience in other plants rather than a practical

310   validation on their suitability in the genus. The selection of reference gene(s) might influence the

311   results or even conclusion of studies, especially those concerning plant development or stress

312   response, as there would be more variation in gene expression under those conditions. As a

313   commonly used reference gene, the *ACT* gene family associated with cell structure maintenance

314   was previously used as the reference gene for normalization in *Lilium* (Wang et al. 2009; Xin et

315   al. 2010), and it showed stable expression in *L. davidii* var. *unicolor* (Li et al. 2015). Nevertheless,

316   in our study, *ACT* failed to be ranked as a satisfactory reference gene under most of the

317   experimental conditions except biotic stress, and even yielded the poorest values during drought

318   and salinity stress. On the other hand, *18S rRNA* was previously proved to be one of the best

319   reference genes for *L. brownii* and some other plant species (Kianianmomeni & Hallmann 2013;

320   Luo et al. 2014; Saha & Vandemark 2013), but in our study, *18S rRNA* was generally evaluated as

321   the one of the least stable reference genes except under drought stress. This is possibly caused by

322   their genotype differences. Some studies on other species also recognized it as an unstable gene

323   (Castro et al. 2012; Qi et al. 2010). *GAPDH* was used for normalization in some gene expression

324   studies concerning the resistance of *L. regale* to *Fusarium oxysporum* (He et al. 2014; Li et al.

325   2014), but our study revealed that it was not stable enough after the plant was inoculated with

326   another important pathogenic fungus, *B. elliptica*, despite its good stability under abiotic stress.

327 Therefore, it would be helpful for future expression studies on *Lilium*, particularly *L. regale*, to
328 take other reference genes into consideration apart from these regular choices, as our study
329 suggested.

330   In general, *BHLH* was recognized as one of the most stably expressed reference genes in all
331 the samples and experimental treatments, but interestingly, it failed to have a good performance
332 under specific conditions. A similar result concerning reference gene *psaA* was also found in the
333 study on chrysanthemum (Gu et al. 2011). As a gene encoded a helicase, basic helix-loop-helix
334 family protein, *BHLH* was reported to have a stable expression in model plants such as *Arabidopsis*
335 *thaliana* (Czechowski et al. 2005) and *Medicago truncatula* (Kakar et al. 2008). Because there is
336 actually not an absolutely stable reference gene in all cases, *BHLH* might be a compromise choice
337 rather than a satisfying one. The normalization of *LrLOX* also confirmed that the result using
338 *BHLH* was not so accurate as those using the recommended genes. It also should be noted that
339 *SAND* acted as a stably but quite lowly expressed gene when compared with other reference genes
340 (Fig. 1). *SAND* might be therefore recommended for the quantification of low abundance
341 transcripts such as transcription factors, so that the difference in quantification cycle (ΔCq) values
342 between the reference transcript and the gene of interest will then be much smaller, and the results
343 will be less influenced by variations in amplification efficiencies and hence more accurate.

344   As mentioned above, the most suitable genes still varied significantly depending on
345 experimental conditions. In the present study, *EF1* was suggested as the most stable reference
346 genes during development in lily leaves, whereas it is reported that *EF1* is unsuitable for
347 normalization at different developmental stages in oil palm (*Elaeis guineensis*) (Yeap et al. 2014).
348 For biotic stress, *SAND* was indicated as the most stable reference genes by all the three algorithms.
349 A combination of two reference genes, *CLA/SAND*, comprised the optimum set under biotic stress.
350 Studies on *Humulus lupulus* and *Citrus* also revealed a stable expression level of *SAND* (Mafra et
351 al. 2012; Stajner et al. 2013), but our result is different from the work on lentil (*Lens culinaris*)
352 and soybean (*Glycine max*), in which *18S rRNA* and *Actin* were the best ones (Ma et al. 2013; Saha

353   & Vandemark 2013). According to our results, *CYP* and *TIP41* were considered to express

354   constantly under drought and salinity stress respectively, and *GADPH* was also relatively stable

355   under both the stresses. Under cold stress condition, a previous study on lentil showed that *GAPDH*

356   was the least stable gene (Saha & Vandemark 2013), but in our study on *Lilium, GAPDH* was

357   calculated as the one of the most stable genes. On the other hand, *GAPDH* was expressed at a more

358   constant level during heat stress treatment in *Chrysanthemum* (Gu et al. 2011), but *CYP* would be

359   a better choice for *Lilium* according the combination of three algorithms. Overall, our results

360   emphasize the importance to identify the most suitable reference genes for individual organisms

361   and different experimental conditions.

362   Lipoxygenases (LOXs), a family of non-heme-iron-containing fatty acid dioxygenases, play

363   a crucial role in lipid peroxidation processes during plant defense responses to biotic stresses such

364   as pathogen infection (Hwang & Hwang 2010). LOXs catalyze the oxidation of polyunsaturated

365   fatty acids into oxylipins, which are then enzymatically metabolized into traumatin, jasmonic acid

366   (JA) and methyl jasmonate (MeJA) (Joo & Oh 2012). These compounds are considered to respond

367   to plant development, senescence and diverse stresses. To evaluate the reference genes selected in

368   this study, we analyzed the expression level of *LrLOX* in the leaves of *L. regale* infected with *B.*

369   *elliptica*. The expression model of *LrLOX* in this study was concordant with that of *PvLOX6* in

370   *Phaseolus vulgaris* after infected with *Pseudomonas syringae* (Porta et al. 2008). The expression

371   profile of *LrLOX* was hardly considered to be reliable when referring to the unstable genes

372   concluded under biotic stress. Our results showed that reference genes would have a severe impact

373   on the expression level of the gene of interest, and the selection of them should be based on a

374   careful and comprehensive evaluation. Therefore, our study should be a new start for the gene

375   expression-based studies on *L. regale*, and would be beneficial for the molecular breeding of

376   *Lilium* in the future.

377   **Conclusion**

378   In summary, we evaluated the expression stability of nine candidate reference genes in *L.*

379 *regale* under different developmental stages, biotic and abiotic stresses to identify the stable one(s)

380 for normalization in gene expression studies. Based on the combination of the analyses by three

381 algorithms, *BHLH* was considered as the most generally stably expressed reference genes under

382 all the experimental conditions, but specific selection still depended on certain experimental

383 environment. The selection of reference genes under biotic stress was further validated in the

384 investigation on the expression pattern of *LrLOX*. With the results outlined in this study, it would

385 be more feasible to implement a sensitive and accurate quantification of gene expression in *L.*

386 *regale*.

387

388 **Acknowledgments:** We would like to thank Miss Yu-Qian Zhao and Miss Xue Gao for their

389 helping in our study.

390

References

392 Andersen C, Jensen J, and Orntoft T. 2004. Normalization of real-time quantitative reverse

393   transcription-PCR data: a model-based variance estimation approach to identify genes

394   suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 64:5245

395   - 5250.

396 Bin WS, Wei LK, Ping DW, Li Z, Wei G, Bing LJ, Gui PB, Jian WH, and Feng CJ. 2012.

397   Evaluation of appropriate reference genes for gene expression studies in pepper by

398   quantitative real-time PCR. *Molecular Breeding* 30:1393-1400.

399 Castro P, Roman B, Rubio J, and Die JV. 2012. Selection of reference genes for expression studies

400   in *Cicer arietinum* L.: analysis of *cyp81E3* gene expression against *Ascochyta rabiei*.

401   *Molecular Breeding* 29:261-274.

402 Czechowski T, Stitt M, Altmann T, Udvardi M, and Scheible W. 2005. Genome-wide

403   identification and testing of superior reference genes for transcript normalization in

404   Arabidopsis. *Plant Physiol* 139:5 - 17.

405   De Spiegelaere W, Dern-Wieloch J, Weigel R, Schumacher V, Schorle H, Nettersheim D,
406   Bergmann M, Brehm R, Kliesch S, and Vandekerckhove L. 2015. Reference Gene
407   Validation for RT-qPCR, a Note on Different Available Software Packages. *PLoS One* 10.

408   Dheda K, Huggett J, Bustin S, Johnson M, Rook G, and Zumla A. 2004. Validation of
409   housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques*
410   37:112 - 114.

411   Fernandez P, Di Rienzo J, Moschen S, Dosio GA, Aguirrezábal LN, Hopp HE, Paniego N, and
412   Heinz R. 2011. Comparison of predictive methods and biological validation for qPCR
413   reference genes in sunflower leaf senescence transcript analysis. *Plant cell reports* 30:63-
414   74.

415   Fu J, Wang Y, Huang H, Zhang C, and Dai S. 2012. Reference gene selection for RT-qPCR
416   analysis of *Chrysanthemum lavandulifolium* during its flowering stages. *Molecular*
417   *Breeding* 31:205-215.

418   Gachon C, Mingam A, and Charrier B. 2004. Real-time PCR: what relevance to plant studies? *J*
419   *Exp Bot* 55:1445 - 1454.

420   Ginzinger D. 2002. Gene quantification using real-time quantitative PCR: an emerging technology
421   hits the mainstream. *Exp Hematol* 30:503 - 512.

422   Gu C, Chen S, Liu Z, Shan H, Luo H, Guan Z, and Chen F. 2011. Reference Gene Selection for
423   Quantitative Real-Time PCR in Chrysanthemum Subjected to Biotic and Abiotic Stress.
424   *Molecular Biotechnology* 49:192-197.

425   Gutierrez L, Mauriat M, Pelloux J, Bellini C, and Van Wuytswinkel O. 2008. Towards a systematic
426   validation of references in real-time rt-PCR. *Plant Cell* 20:1734 - 1735.

427   He H, Liu D, Zhang N, Zheng W, Han Q, Ji B, Ge F, and Chen C. 2014. The PR10 gene family is
428   highly expressed in *Lilium regale* Wilson during *Fusarium oxysporum* f. sp. *lilii* infection.
429   *Genes & genomics* 36:497-507.

430   Hwang IS, and Hwang BK. 2010. The pepper 9-lipoxygenase gene *CaLOX1* functions in defense

431   and cell death responses to microbial pathogens. *Plant Physiology* 152:948-967.

432   Jiang Q, Wang F, Li M-Y, Ma J, Tan G-F, and Xiong A-S. 2014. Selection of suitable reference

433   genes for qPCR normalization under abiotic stresses in *Oenanthe javanica* (BI.) DC. *PLoS*

434   *One* 9.

435   Joo Y-C, and Oh D-K. 2012. Lipoxygenases: potential starting biocatalysts for the synthesis of

436   signaling compounds. *Biotechnology advances* 30:1524-1532.

437   Kakar K, Wandrey M, Czechowski T, Gaertner T, Scheible W-R, Stitt M, Torres-Jerez I, Xiao Y,

438   Redman JC, and Wu HC. 2008. A community resource for high-throughput quantitative

439   RT-PCR analysis of transcription factor gene expression in *Medicago truncatula*. *Plant*

440   *Methods* 4:18.

441   Kianianmomeni A, and Hallmann A. 2013. Validation of reference genes for quantitative gene

442   expression studies in *Volvox carteri* using real-time RT-PCR. *Molecular biology reports*

443   40:6691-6699.

444   Li H, Liu D, He H, Zhang N, Ge F, and Chen C. 2014. Molecular cloning of a 14-3-3 protein gene

445   from *Lilium regale* Wilson and overexpression of this gene in tobacco increased resistance

446   to pathogenic fungi. *Scientia Horticulturae* 168:9-16.

447   Li X, Cheng J, Zhang J, da Silva JAT, Wang C, and Sun H. 2015. Validation of Reference Genes

448   for Accurate Normalization of Gene Expression in *Lilium davidii* var. *unicolor* for Real

449   Time Quantitative PCR. *PLoS One* 10:e0141323.

450   Luo H, Luo L, Guan B, Li E, Xiong D, Sun B, Peng K, and Yang B. 2014. Evaluation of candidate

451   reference genes for RT-qPCR in lily (*Lilium brownii*). *The Journal of Horticultural Science*

452   *and Biotechnology* 89:345-351.

453   Ma SH, Niu HW, Liu CJ, Zhang J, Hou CY, and Wang DM. 2013. Expression Stabilities of

454   Candidate Reference Genes for RT-qPCR under Different Stress Conditions in Soybean.

455   *PLoS One* 8.

456   Mafra V, Kubo KS, Alves-Ferreira M, Ribeiro-Alves M, Stuart RM, Boava LP, Rodrigues CM,

457   and Machado MA. 2012. Reference Genes for Accurate Transcript Normalization in Citrus

458   Genotypes under Different Experimental Conditions. *PLoS One* 7:e31263.

459   Nicot N. 2005. Housekeeping gene selection for real-time RT-PCR normalization in potato during

460   biotic and abiotic stress. *Journal of Experimental Botany* 56:2907-2914.

461   Pfaffl MW, Tichopad A, Prgomet C, and Neuvians TP. 2004. Determination of stable

462   housekeeping genes, differentially regulated target genes and sample integrity:

463   BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnology letters* 26:509-

464   515.

465   Porta H, Figueroa-Balderas RE, and Rocha-Sosa M. 2008. Wounding and pathogen infection

466   induce a chloroplast-targeted lipoxygenase in the common bean (*Phaseolus vulgaris* L.).

467   *Planta* 227:363-373.

468   Qi J, Yu S, Zhang F, Shen X, Zhao X, Yu Y, and Zhang D. 2010. Reference Gene Selection for

469   Real-Time Quantitative Polymerase Chain Reaction of mRNA Transcript Levels in

470   Chinese Cabbage (*Brassica rapa* L. ssp. *pekinensis*). *Plant Molecular Biology Reporter*

471   28:597-604.

472   Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, and Nitsche A. 2004. Guideline to

473   reference gene selection for quantitative real-time PCR. *Biochemical and biophysical*

474   *research communications* 313:856-862.

475   Saha GC, and Vandemark GJ. 2013. Stability of Expression of Reference Genes Among Different

476   Lentil (*Lens culinaris*) Genotypes Subjected to Cold Stress, White Mold Disease, and

477   Aphanomyces Root Rot. *Plant Molecular Biology Reporter* 31:1109-1115.

478   Schönbach C, Chandna R, Augustine R, and Bisht NC. 2012. Evaluation of Candidate Reference

479   Genes for Gene Expression Normalization in *Brassica juncea* Using Real Time

480   Quantitative RT-PCR. *PLoS One* 7:e36918.

481   Stajner N, Cregeen S, and Javornik B. 2013. Evaluation of Reference Genes for RT-qPCR

482   Expression Studies in Hop (*Humulus lupulus* L.) during Infection with Vascular Pathogen

483   *Verticillium albo-atrum*. *PLoS One* 8:e68228.

484 Tong Z, Li QH, Yang YJ, Dai FW, Gao JP, and Hong B. 2013. Isolation and expression analysis

485   of *LoPIP2*, a lily (*Lilium* Oriental Hybrids) aquaporin gene involved in desiccation-induced

486   anther dehiscence. *Scientia Horticulturae* 164:316-322.

487 Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, and Speleman F. 2002.

488   Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of

489   multiple internal control genes. *Genome Biol* 3:1-12.

490 Wang A, Tang J, Li D, Chen C, Zhao X, and Zhu L. 2009. Isolation and functional analysis of

491   *LiYAB1*, a *YABBY* family gene, from lily (*Lilium longiflorum*). *Journal of plant physiology*

492   166:988-995.

493 Xin H, Zhang H, Chen L, Li X, Lian Q, Yuan X, Hu X, Cao L, He X, and Yi M. 2010. Cloning

494   and characterization of *HsfA2* from Lily (*Lilium longiflorum*). *Plant cell reports* 29:875-

495   885.

496 Yeap WC, Loo JM, Wong YC, and Kulaveerasingam H. 2014. Evaluation of suitable reference

497   genes for qRT-PCR gene expression normalization in reproductive, vegetative tissues and

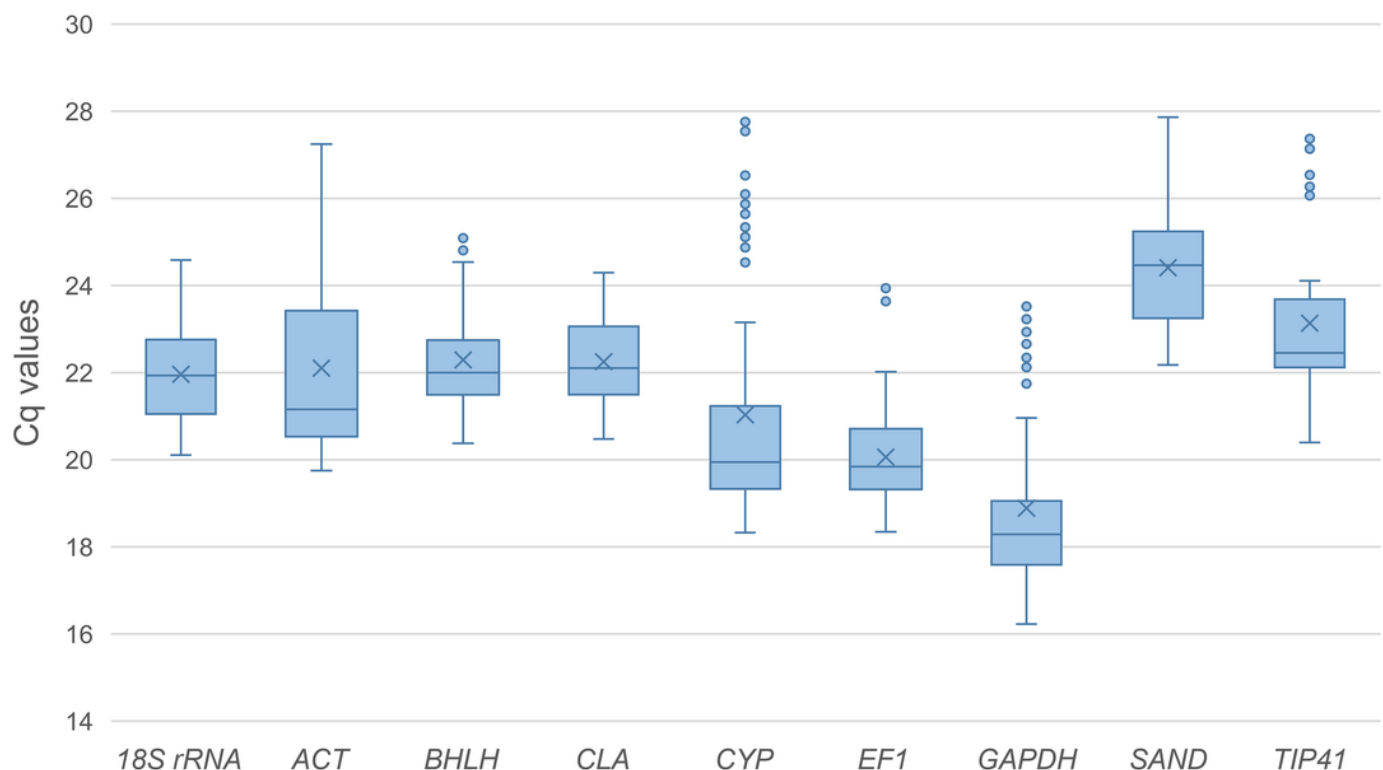
498   during fruit development in oil palm. *Plant Cell Tissue and Organ Culture* 116:55-66.

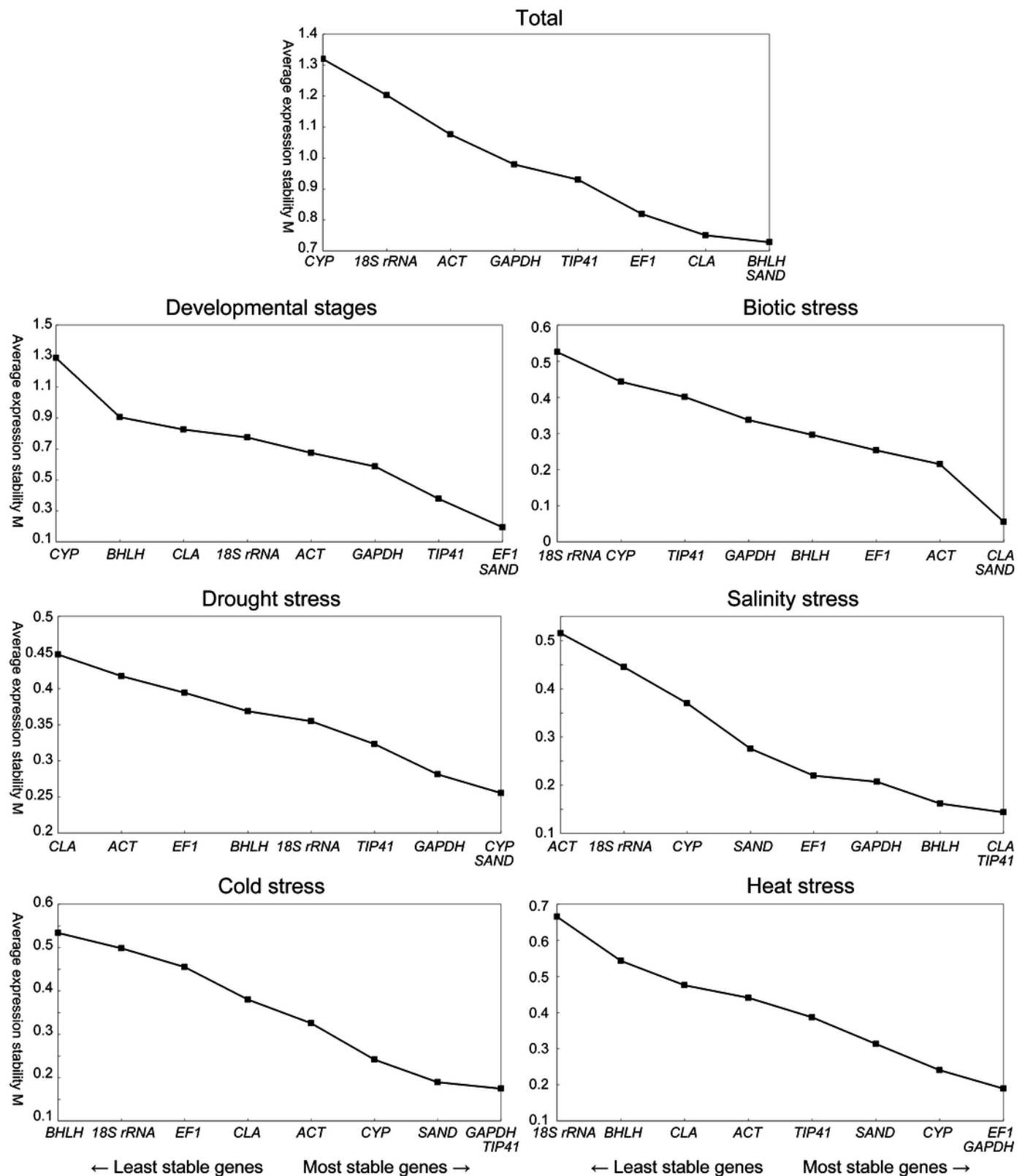
499 Zhao X, Chen X, Li D, and Liu K. 1994. Resources and research situation of the genus *Lilium* in

500   China. International Symposium on the Genus *Lilium* 414. Taejon, Korea. p 59-68.

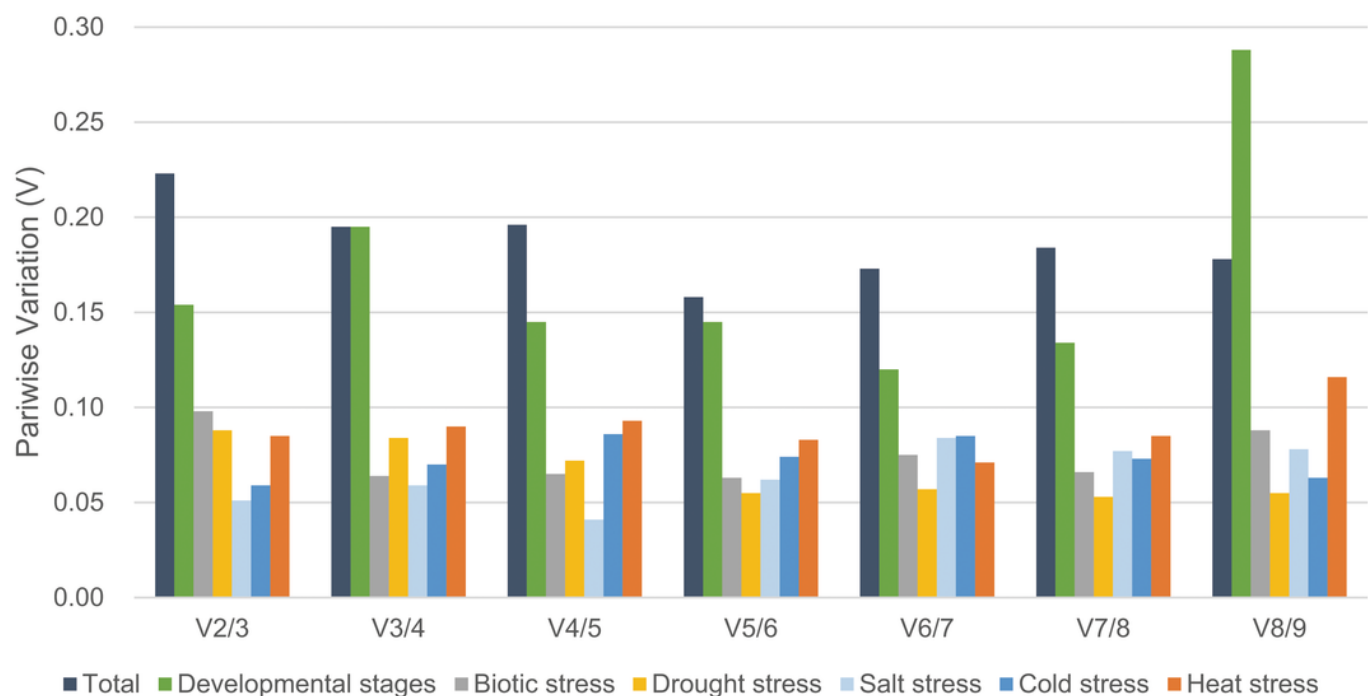
1

Quantification cycle (Cq) values of the nine candidate reference genes across all the experimental samples.

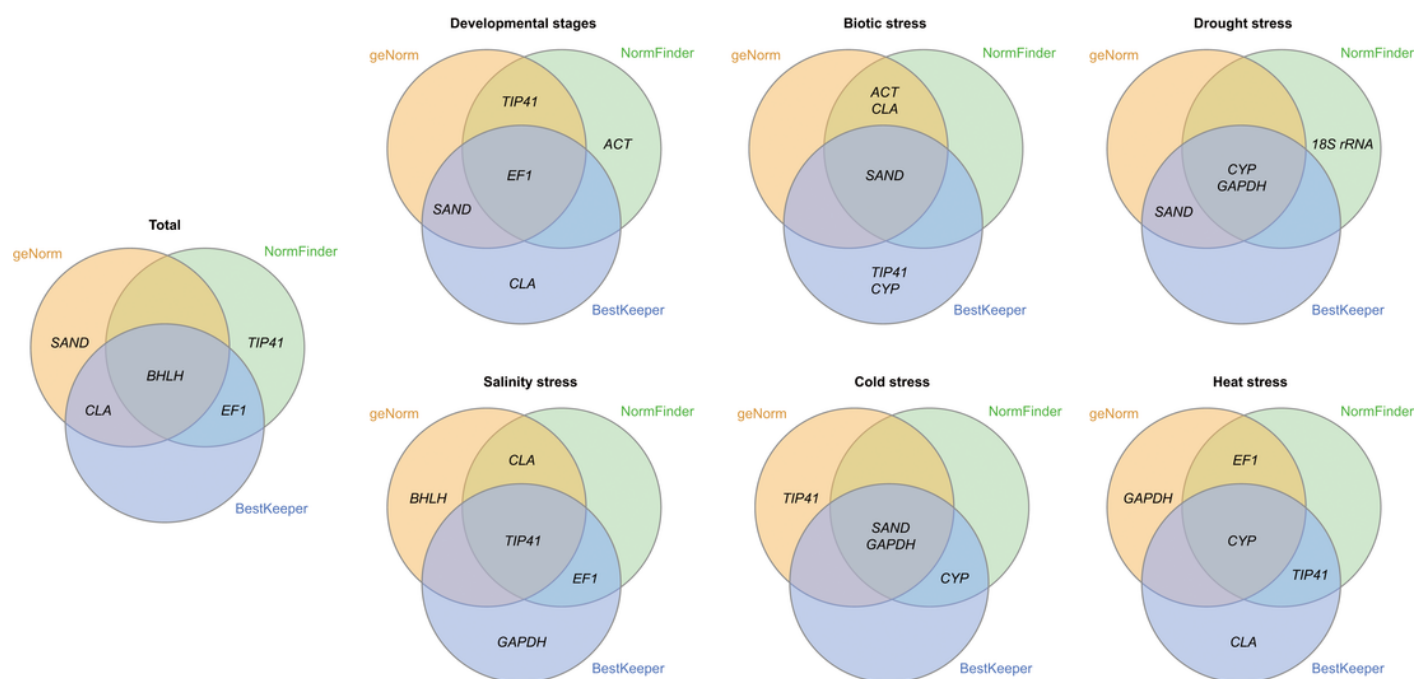
Whiskers represent the local maximum and minimum values, and points beyond the end of each whisker mark the outliers. Lower and upper boxes indicate the 25th to 75th percentile, and the midlines inside the boxes represent the medians. The means are denoted by "×" markers.

Average expression stability and ranking of the nine candidate reference genes calculated by geNorm.

A lower value of average expression stability (M) indicates more stable expression. The least stable genes are on the left and the most stable genes on the right.

3

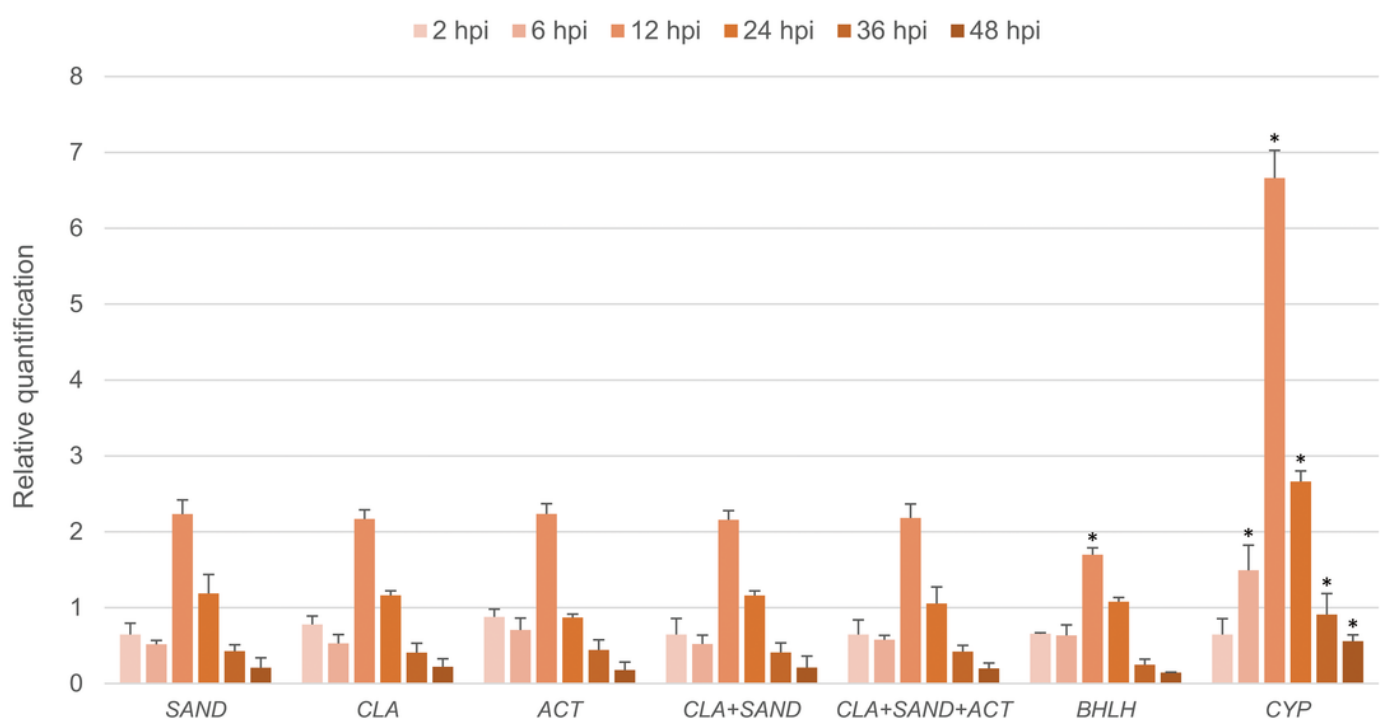
Determination of the optimal number of reference genes by pairwise variation using geNorm.

Pairwise variation (V_n/V_{n+1}) was analyzed between the normalization factors NF_n and NF_{n+1}, carried out for all the samples (Total) and the samples at different developmental stages or under different stresses.

4

Venn diagram showing the most stable genes identified by geNorm, NormFinder and BestKeeper.

Three most stable genes selected by each algorithm are presented.

5

Relative quantification of *LrLOX* in leaves at different time points after inoculation with *B. elliptica*.

Asterisks (*) indicate significant differences (p < 0.05) relative to the value normalized with *SAND* at each time point.

Characteristics of the nine candidate reference genes.

1 **Table 1.** Characteristics of the nine candidate reference genes

Gene	Full name	Accession number	Cellular function
18S rRNA	*18S ribosomal RNA*	HQ686070	Ribosome subunit
ACT	*Beta Actin*	KJ543460	Cytoskeletal structural protein
BHLH	*Basic helix-loop-helix*	KJ543467	Transcription factor
CLA	*Clathrin*	KJ543465	Clathrin adaptor complex
CYP	*Cycolphilin A*	KJ543464	Serine-threonine phosphatase inhibitor
EF1	*Elongation factor 1-a*	KJ543461	Eukaryotic elongation factor 1
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*	KJ543468	Oxidoreductase in glycolysis and gluconeogenesis
SAND	*SAND family protein*	KJ543463	Hypothetical proteins
TIP41	*TIP41 family protein*	KJ543466	Tonoplast intrinsic proteins

Primer sequences and optimized reaction conditions of the nine candidate reference genes.

Note: F: Forward, R: Reverse. T$_a$: Annealing temperature.

1 **Table 2.** Primer sequences and optimized reaction conditions of the nine candidate reference genes

Gene	Prime sequence (5′–3′)	Amplicon size (bp)	Primer concentration (μM)	T_a (°C)	Amplicon T_m (°C)	PCR efficiency (%)	Regression coefficient (R^2)
18S rRNA	F: GCAGAATCCCGTGAACCAT	143	0.3	54	87.8	93.7	0.998
	R: GCCAATCTCCGCATCCAT						
ACT	F: CCCATTGAGCACGGCATTGTC	128	0.2	56	85.7	102.8	0.999
	R: GGATTGAGAGGAGCTTCGGTGAGA						
BHLH	F: CCAGCAGGTTGTCCTTGTG	142	0.3	56	85.1	97.5	0.998
	R: TCCGTGATGAGAAGCAGAGG						
CLA	F: GATGAGATTCTGATTGCTGGTGAG	103	0.3	55	84.2	93	0.999
	R: CCTGCTCTTTGGCTGTTTCC						
CYP	F: ACCCTTGGGCAAGAACAACAGAA	127	0.3	56	84.3	99.3	0.997
	R: GCAAAGGAGGTTGAGTTGGAGGAT						
EF1	F: GGCACTAACTCGCTCCTTCTG	173	0.2	55	84.6	101.6	0.998
	R: TTGGTAAGATGCTGGTGATTGGAT						
GAPDH	F: CACGGTCAGTGGAAGCACCATGAGAT	180	0.3	60	86.3	97.6	0.995
	R: AGCAGCAGCCTTATCCTTGTCAGTGA						
SAND	F: CCAATACCCAGATGAGGAGACAAA	178	0.1	54	83.8	105.4	0.992
	R: GGATTCGCATTGAGGCTGTTC						
TIP41	F: CGAAGCCAGAAACGGAGAAGAAT	192	0.3	55	82.9	93.6	0.998
	R: GGGTAGGGTGGATTGGGAAGA						

2 Note: F: Forward, R: Reverse. T_a: Annealing temperature. Amplicon T_m: Predicted melting temperature of amplicons.

Gene expression stability of the nine candidate reference genes under different conditions calculated by NormFinder.

Note: A lower normalization factor (NF) value indicates higher stability of the gene.

1 **Table 3.** Gene expression stability of the nine candidate reference genes under different conditions calculated by NormFinder

Total		Developmental stages		Biotic stress		Drought stress		Salinity stress		Cold stress		Heat stress	
Gene	NF	Gene	NF	Gene	NF	Gene	NF	Gene	NF	Gene	NF	Gene	NF
TIP41	0.382	*TIP41*	0.158	*CLA*	0.058	*CYP*	0.117	*TIP41*	0.054	*SAND*	0.052	*CYP*	0.073
EF1	0.413	*ACT*	0.268	*SAND*	0.073	*18S rRNA*	0.139	*EF1*	0.073	*CYP*	0.090	*TIP41*	0.164
BHLH	0.436	*EF1*	0.269	*ACT*	0.096	*GAPDH*	0.145	*CLA*	0.074	*GAPDH*	0.115	*EF1*	0.206
SAND	0.469	*SAND*	0.331	*EF1*	0.154	*TIP41*	0.177	*GAPDH*	0.083	*TIP41*	0.173	*CLA*	0.240
GAPDH	0.483	*GAPDH*	0.414	*BHLH*	0.215	*BHLH*	0.199	*BHLH*	0.092	*EF1*	0.329	*GAPDH*	0.282
CLA	0.657	*18S rRNA*	0.470	*GAPDH*	0.218	*SAND*	0.258	*SAND*	0.270	*CLA*	0.348	*ACT*	0.315
ACT	0.768	*CLA*	0.620	*TIP41*	0.353	*EF1*	0.265	*CYP*	0.418	*ACT*	0.349	*SAND*	0.362
18S rRNA	1.068	*BHLH*	0.915	*CYP*	0.419	*ACT*	0.311	*18S rRNA*	0.437	*BHLH*	0.383	*BHLH*	0.455
CYP	1.085	*CYP*	1.789	*18S rRNA*	0.543	*CLA*	0.333	*ACT*	0.478	*18S rRNA*	0.385	*18S rRNA*	0.717

2 Note: A lower normalization factor (NF) value indicates higher stability of the gene.

Descriptive statistics and stability values of the nine candidate reference genes under different conditions calculated by BestKeeper.

Note: A SD [± CP] value below 1 and a SD [± x-fold] value below 2, marked in green, suggest that the reference gene is qualified for normalization. The values of correlation coefficient (r) were used for ranking in this study.

1 **Table 4.** Descriptive statistics and stability values of the nine candidate reference genes under different conditions calculated by

2 BestKeeper

		18S rRNA	ACT	BHLH	CLA	CYP	EF1	GAPDH	SAND	TIP41
Total	SD [± CP]	0.794	1.826	0.982	0.799	2.048	0.866	1.508	1.045	1.374
	SD [± x-fold]	1.734	3.546	1.975	1.740	4.135	1.823	2.844	2.064	2.592
	coeff. of corr. [r]	0.356	0.958	0.885	0.766	0.921	0.904	0.970	0.871	0.966
Developmental stages	SD [± CP]	1.205	1.643	0.702	0.690	3.190	0.988	1.690	0.919	1.258
	SD [± x-fold]	2.305	3.122	1.626	1.613	9.126	1.984	3.226	1.891	2.391
	coeff. of corr. [r]	0.909	0.993	0.693	0.899	0.940	0.967	0.967	0.952	0.996
Biotic stress	SD [± CP]	0.257	0.334	0.235	0.353	0.604	0.261	0.376	0.366	0.594
	SD [± x-fold]	1.195	1.261	1.177	1.277	1.520	1.198	1.298	1.288	1.509
	coeff. of corr. [r]	-0.552	0.909	0.625	0.930	0.955	0.792	0.780	0.931	0.963
Drought stress	SD [± CP]	0.140	0.429	0.267	0.405	0.361	0.196	0.413	0.492	0.405
	SD [± x-fold]	1.102	1.347	1.204	1.325	1.285	1.146	1.332	1.406	1.324
	coeff. of corr. [r]	0.904	0.636	0.740	0.544	0.944	0.408	0.944	0.977	0.859
Salinity stress	SD [± CP]	0.458	0.945	0.494	0.530	0.621	0.508	0.574	0.509	0.599
	SD [± x-fold]	1.373	1.925	1.408	1.444	1.538	1.422	1.488	1.423	1.514
	coeff. of corr. [r]	0.603	0.974	0.964	0.966	0.762	0.975	0.975	0.850	0.977
Cold stress	SD [± CP]	0.223	0.627	0.560	0.555	0.397	0.189	0.396	0.444	0.429
	SD [± x-fold]	1.167	1.544	1.474	1.469	1.317	1.140	1.316	1.360	1.346
	coeff. of corr. [r]	0.005	0.821	0.778	0.875	0.936	0.328	0.930	0.972	0.898
Heat stress	SD [± CP]	1.005	0.369	0.529	0.512	0.209	0.141	0.112	0.347	0.355
	SD [± x-fold]	2.008	1.291	1.443	1.426	1.156	1.102	1.080	1.272	1.279
	coeff. of corr. [r]	0.846	0.511	0.382	0.972	0.937	0.378	-0.412	0.223	0.786

3 Note: A SD [± CP] value below 1 and a SD [± x-fold] value below 2, marked in green, suggest that the reference gene is qualified for

4 normalization. The values of correlation coefficient (r) were used for ranking in this study.