Selection of reference genes for qPCR normalization in buffalobur (Solanum rostratum Dunal)

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Buffalobur (Solanum rostratum Dunal), which belongs to the Solanaceae family, is a worldwide noxious invasive weed and is listed as one of the top 10 alien invasive species in China. It is harmful to humans and livestock because the entire plant is covered with spines containing toxins. Many studies have analysed the gene expression in this weed species under different stress conditions using quantitative real-time PCR (qPCR). However, until now, there has been no report on suitable reference genes in buffalobur. Herein, 14 candidate reference genes were selected and evaluated for their expression stability in buffalobur in different tissues, at different developmental stages, and in response to several stress conditions using the geNorm, NormFinder, BestKeeper and RefFinder statistical algorithms. The results showed that EF1α, ACT and SAND are suitable reference genes across all samples tested. We recommend the normalization of target gene expression under different experimental conditions using these three genes together. Validation of selected reference genes was achieved by assessing the relative expression levels of P5CS and GI. This work identified the appropriate reference genes for transcript normalization in buffalobur, which will be helpful in future genetic studies of this invasive weed.

Buffalobur (Solanum rostratum Dunal) is native to North America and is ranked as a highly invasive weed species across the world¹. Buffalobur poses a serious threat to local biodiversity and agro-ecosystems. The aggressive growth of this weed results in devastating damage to crop production². Additionally, buffalobur is a crucial intermediate host for a wide range of pests and diseases, which threaten the health of crops³. Moreover, this weed species is harmful to humans and livestock because its leaves, stems and calyx are covered with spines containing toxins³. Eradication of this weed using conventional control measures including manual, mechanical, or chemical methods is difficult. Therefore, new and innovative approaches are being explored to control this weed. Measuring gene expression will help form weed-control approaches, and quantitative gene expression measurement requires appropriate reference genes.

Due to its advantages of high sensitivity and specificity, qPCR has been widely used to quantify gene expression to discover the genetic basis of physiological patterns during the plant life cycle⁴. Attaining precision in qPCR-based analysis depends on the selection of a suitable reference gene in experimental sets⁵. The expression level of the appropriate internal control gene should remain relatively constant and should not change significantly across experimental conditions, types of tissues, developmental stages or stress treatments⁶; however, in practice, no gene exhibits fully stable expression throughout all growth stages and experimental conditions. It has been suggested that multiple reference genes can achieve accurate normalization⁷. There is general agreement that the expression stability of candidate genes should be validated prior to initiating normalization studies using qPCR in a particular species.

There have been studies on the selection and validation of reference genes in many Solanaceae plants, such as pepper (Capsicum annuum L.)⁹, potato (Solanum tuberosum L.)¹⁰, eggplant (Solanum melongena L.)¹¹, tomato (Lycopersicon esculentum Mill.)¹², Lycium barbarum L. and L. ruthenicum Murray¹³. However, until now, no appropriate reference gene has been identified for qPCR analysis in buffalobur. In this study, 14 genes, namely, GAPDH, ACT, GR, UBQ, TIP41, RPL8, aIF, DNAJ, TUB, CYP, EF1α, PP2Ac, RUBP, and SAND, were selected as

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candidate reference genes for buffalobur. The expression stabilities of these genes were tested with respect to different developmental periods, tissues, abiotic stresses, and hormone stimuli and with glyphosate treatment using geNorm, NormFinder, BestKeeper and RefFinder to identify the most stable gene for qPCR normalization in buffalobur.

Table 1. Information on the selected primer pairs and amplification characteristics of the 14 candidate reference genes and 2 target genes for buffalobur.
normalization because their V2/3 values were all less than 0.15 (see Supplementary Fig. S2).)

According to the Cq values, we can make a preliminary judgement that RUBP in roots were relatively high. In both the abiotic stress and glyphosate group. For the hormone stimuli group, the best gene pairs were ACT and PP2Acs (Tables 2, 3 and 4; see Supplementary Fig. S1). For leaves of different developmental stages and different tissues harvested in the fruiting period.

Additionally, geNorm provides the pairwise variation (Vn/Vn+1), which determines the minimal number of reference genes to obtain an accurate and reliable normalization. A value of 0.2 is usually considered acceptable. For the total samples group, the V3/4 value of 0.186 indicated that the top three reference genes (ACT, PP2Acs and SAND) would be appropriate to use for normalization; for the other groups, the top two genes were sufficient for normalization because their V2/3 values were all less than 0.15 (see Supplementary Fig. S2).

### Table 2. Gene expression stability ranked by four algorithms for the “Developmental stages” and “Tissues” groups. (The fully expanded leaves were harvested at different developmental stages. Different tissues were harvested in the fruiting period.

| Group         | Rank | geNorm Gene | Stability | NormFinder Gene | Stability | BestKeeper Gene | SD [±Cq] | CV (%Cq) | RefFinder Gene | Stability |
|---------------|------|-------------|-----------|----------------|-----------|----------------|---------|----------|----------------|-----------|
| **Developmental stages** | | | | | | | |
| SAND          | 1    | eIF         | 0.343     | ACT            | 0.084     | GR             | 0.30    | 1.24     | ACT            | 2.11      |
| UBQ           | 2    | SAND        | 0.343     | EF1α           | 0.124     | ACT            | 0.35    | 1.80     | EF1α           | 2.51      |
| EF1α          | 3    | UBQ         | 0.411     | GR             | 0.185     | PP2Acs         | 0.38    | 1.73     | GR             | 2.82      |
| ACT           | 4    | EF1α        | 0.530     | PP2Acs         | 0.287     | CYP            | 0.44    | 2.51     | SAND           | 3.66      |
| PP2Acs        | 5    | ACT         | 0.576     | CYP            | 0.295     | EF1α           | 0.48    | 2.70     | PP2Acs         | 4.56      |
| SAND          | 6    | PP2Acs      | 0.610     | SAND           | 0.359     | SAND           | 0.58    | 2.45     | CYP            | 5.03      |
| UBQ           | 7    | GR          | 0.654     | UBQ            | 0.425     | UBQ            | 0.61    | 3.10     | eIF            | 5.20      |
| CYP           | 8    | GR          | 0.654     | UBQ            | 0.425     | UBQ            | 0.61    | 3.10     | eIF            | 5.20      |
| GAPDH         | 9    | GAPDH       | 0.715     | eIF            | 0.522     | eIF            | 0.76    | 3.52     | GAPDH          | 7.97      |
| RPL8          | 10   | RPL8        | 0.747     | RPL8           | 0.564     | RPL8           | 0.83    | 4.03     | RPL8           | 10.00     |
| TUB           | 11   | UBQ         | 0.794     | TUB            | 0.714     | RUBP           | 0.88    | 6.73     | TUB            | 11.24     |
| TIP41         | 12   | TIP41       | 0.929     | TIP41          | 1.040     | TUB            | 1.11    | 5.49     | TIP41          | 12.24     |
| RUBP          | 13   | RUBP        | 1.039     | RUBP           | 1.048     | TIP41          | 1.14    | 5.20     | RUBP           | 12.47     |
| DNAJ          | 14   | DNAJ        | 1.260     | DNAJ           | 1.724     | DNAJ           | 1.99    | 9.98     | DNAJ           | 14.00     |
| **Tissues**   | | | | | | | |
| SAND          | 1    | eIF         | 0.413     | SAND           | 0.166     | PP2Acs         | 0.40    | 1.89     | SAND           | 1.57      |
| UBQ           | 2    | SAND        | 0.413     | PP2Acs         | 0.181     | SAND           | 0.45    | 1.94     | PP2Acs         | 1.93      |
| CYP           | 3    | CYP         | 0.459     | CYP            | 0.289     | CYP            | 0.54    | 3.07     | eIF            | 2.51      |
| EF1α          | 4    | EF1α        | 0.532     | eIF            | 0.364     | GR             | 0.56    | 2.26     | CYP            | 3.22      |
| RPL8          | 5    | RPL8        | 0.574     | ACT            | 0.579     | eIF            | 0.67    | 3.21     | EF1α           | 6.45      |
| GAPDH         | 6    | GAPDH       | 0.601     | GAPDH          | 0.619     | UBQ            | 0.68    | 3.88     | GAPDH          | 6.51      |
| PP2Acs        | 7    | UBQ         | 0.636     | UBQ            | 0.640     | ACT            | 0.72    | 4.14     | ACT            | 6.85      |
| UBQ           | 8    | UBQ         | 0.732     | GR             | 0.643     | EF1α           | 0.87    | 5.00     | UBQ            | 7.20      |
| ACT           | 9    | ACT         | 0.792     | EF1α           | 0.657     | TUB            | 1.01    | 5.17     | GR             | 7.52      |
| GR            | 10   | GR          | 0.843     | RPL8           | 0.726     | GAPDH          | 1.04    | 5.74     | RPL8           | 8.39      |
| TUB           | 11   | TUB         | 0.943     | TUB            | 0.943     | RPL8           | 1.06    | 5.11     | TUB            | 10.46     |
| TIP41         | 12   | TIP41       | 1.084     | TIP41          | 1.206     | DNAJ           | 1.52    | 8.05     | TIP41          | 12.24     |
| DNAJ          | 13   | DNAJ        | 1.330     | DNAJ           | 1.483     | TIP41          | 1.66    | 7.70     | DNAJ           | 12.74     |
| RUBP          | 14   | RUBP        | 1.936     | RUBP           | 3.812     | RUBP           | 3.85    | 20.82    | RUBP           | 14.00     |

Results

Expression profiles of candidate reference genes. The quantification cycle values (Cq) of the 14 candidate genes across all samples ranged from 11.8 to 30.8, showing a wide range of variation (Fig. 1). The majority of values were between 18 and 23. The Cq values of TIP41 and RUBP ranged from 18.8 to 30.8 and from 11.8 to 28.3, respectively, showing great expression differences. Moreover, the Cq values of RUBP in roots were relatively high. According to the Cq values, we can make a preliminary judgement that TIP41 and RUBP might not be suitable candidate reference genes.

geNorm analysis. The geNorm software identifies the optimal reference gene pair; smaller M values correlate with more stable gene expression. Some studies have established the threshold of M values ≤ 1.0 or 1.5 to identify appropriate reference genes17. M values below 0.5 are indicative of strong reference genes. Except for the group of total samples (M = 0.527), the M values of the best pairs in other groups were less than 0.5 in our study (Tables 2, 3 and 4; see Supplementary Fig. S1). For leaves of different developmental stages and different tissues in the fruiting period, the best two gene pairs were eIF and SAND. RPL8 and EF1α were the most stable gene pair in both the abiotic stress and glyphosate group. For the hormone stimuli group, the best gene pairs were ACT and RPL8. For the total samples group, the best gene pairs were ACT and PP2Acs. TIP41, RUBP and DNAJ were low-ranking candidates according to geNorm.

Additionally, geNorm provides the pairwise variation (Vn/Vn+1), which determines the minimal number of reference genes to obtain an accurate and reliable normalization. A value of 0.2 is usually considered acceptable. For the total samples group, the V3/4 value of 0.186 indicated that the top three reference genes (ACT, PP2Acs and SAND) would be appropriate to use for normalization; for the other groups, the top two genes were sufficient for normalization because their V2/3 values were all less than 0.15 (see Supplementary Fig. S2).

NormFinder analysis. The lower the stability value obtained via NormFinder, the more stable the candidate reference gene is. A grouped analysis can be used to evaluate these candidate reference genes with this statistical algorithm. The output includes the best reference gene and the best combination of two reference genes (Tables 2, 3 and 4; see Supplementary Table S3). Notably, the ranking alone cannot identify whether a candidate gene is...
Table 3. Gene expression stability ranked by four algorithms for the "Abiotic stresses", "Hormone stimuli" and "Glyphosate" groups.

good, the stability values need to be considered for the assessment. Only when the stability value is low enough, the gene can be considered good. In our study, for leaves of different developmental stages (grouped by stage), the top three genes were \( eIF1 \) (0.015) and \( GR \) (0.111), and the best combination of genes was \( GR \) and \( eIF \) (0.036). For samples of hormone stimuli (grouped by ABA and GA), \( eIF1 \) (0.015) was also the best reference gene, and \( EF1 \) and \( SAND \) (0.013) formed the best gene pair. For the total samples (grouped by sub-group), the top three genes were \( EF1 \) (0.065), \( ACT \) (0.065) and \( TUB \) (0.072); the best combination of two genes

| Group         | Rank | Gene | geNorm Stability | NormFinder Stability | BestKeeper SD [\( \Delta Cq \)] | CV (%Cq) | RefFinder Stability |
|---------------|------|------|------------------|----------------------|---------------------------------|---------|--------------------|
| Abiotic stresses | 1    | RPL8 | 0.355            | EF1α                 | 0.123                           | 0.42    | 2.43               |
|               | 2    | EF1α | 0.355            | TUB                  | 0.175                           | 0.49    | 2.57               |
|               | 3    | ACT  | 0.413            | ACT                  | 0.201                           | 0.51    | 2.25               |
|               | 4    | TUB  | 0.454            | RPL8                 | 0.207                           | 0.56    | 2.82               |
|               | 5    | PP2Ac | 0.489           | PP2Ac               | 0.245                           | 0.56    | 2.35               |
|               | 6    | SAND | 0.568            | GR                   | 0.442                           | 0.57    | 3.06               |
|               | 7    | GR   | 0.647            | SAND                 | 0.444                           | 0.69    | 5.13               |
|               | 8    | RUBP | 0.721            | RUBP                 | 0.585                           | 0.78    | 3.21               |
|               | 9    | CYP  | 0.780            | eIF                  | 0.605                           | 0.85    | 4.07               |
|               | 10   | GAPDH| 0.865            | eIF                  | 0.780                           | 0.86    | 4.89               |
|               | 11   | eIF  | 0.958            | GAPDH                | 0.880                           | 1.20    | 6.47               |
|               | 12   | UBQ  | 1.069            | DNAJ                 | 1.056                           | 1.24    | 6.45               |
|               | 13   | TIP41| 1.180            | DNAJ                 | 1.211                           | 1.26    | 6.73               |
|               | 14   | DNAJ | 1.283            | TIP41                | 1.225                           | 1.47    | 6.38               |

| Hormone stimuli | Rank | Gene | geNorm Stability | NormFinder Stability | BestKeeper SD [\( \Delta Cq \)] | CV (%Cq) | RefFinder Stability |
|-----------------|------|------|------------------|----------------------|---------------------------------|---------|--------------------|
|                 | 1    | ACT  | 0.250            | SAND                 | 0.061                           | 0.18    | 0.78               |
|                 | 2    | RPL8 | 0.250            | EF1α                 | 0.077                           | 0.20    | 1.22               |
|                 | 3    | EF1α | 0.264            | ACT                  | 0.139                           | 0.28    | 1.42               |
|                 | 4    | GAPDH| 0.281            | UBQ                  | 0.143                           | 0.29    | 1.41               |
|                 | 5    | SAND | 0.300            | RPL8                 | 0.189                           | 0.31    | 1.62               |
|                 | 6    | UBQ  | 0.314            | TUB                  | 0.216                           | 0.34    | 1.78               |
|                 | 7    | TUB  | 0.348            | GAPDH                | 0.224                           | 0.35    | 1.92               |
|                 | 8    | eIF  | 0.374            | eIF                  | 0.254                           | 0.36    | 1.46               |
|                 | 9    | PP2Ac| 0.393            | PP2Ac               | 0.262                           | 0.37    | 1.66               |
|                 | 10   | CYP  | 0.418            | CYP                  | 0.274                           | 0.40    | 2.37               |
|                 | 11   | GR   | 0.443            | GR                   | 0.341                           | 0.40    | 2.25               |
|                 | 12   | RUBP | 0.484            | RUBP                 | 0.413                           | 0.49    | 3.57               |
|                 | 13   | DNAJ | 0.586            | DNAJ                 | 0.804                           | 0.77    | 4.01               |
|                 | 14   | TIP41| 0.697            | TIP41                | 0.906                           | 0.81    | 3.56               |

| Glyphosate      | Rank | Gene | geNorm Stability | NormFinder Stability | BestKeeper SD [\( \Delta Cq \)] | CV (%Cq) | RefFinder Stability |
|-----------------|------|------|------------------|----------------------|---------------------------------|---------|--------------------|
|                 | 1    | RPL8 | 0.363            | TUB                  | 0.088                           | 0.26    | 1.17               |
|                 | 2    | EF1α | 0.363            | ACT                  | 0.129                           | 0.30    | 1.31               |
|                 | 3    | ACT  | 0.436            | RPL8                 | 0.134                           | 0.31    | 1.67               |
|                 | 4    | GAPDH| 0.489            | CYP                  | 0.184                           | 0.46    | 2.67               |
|                 | 5    | CYP  | 0.518            | EF1α                 | 0.194                           | 0.49    | 2.73               |
|                 | 6    | TUB  | 0.544            | GAPDH                | 0.306                           | 0.52    | 2.11               |
|                 | 7    | PP2Ac| 0.611            | PP2Ac               | 0.369                           | 0.56    | 2.75               |
|                 | 8    | SAND | 0.648            | SAND                 | 0.425                           | 0.60    | 2.99               |
|                 | 9    | GR   | 0.687            | GR                   | 0.591                           | 0.61    | 3.05               |
|                 | 10   | eIF  | 0.756            | UBQ                  | 0.668                           | 0.64    | 3.41               |
|                 | 11   | UBQ  | 0.792            | eIF                  | 0.701                           | 0.67    | 3.58               |
|                 | 12   | DNAJ | 0.971            | RUBP                 | 1.299                           | 1.39    | 7.28               |
|                 | 13   | RUBP | 1.156            | DNAJ                 | 1.437                           | 1.78    | 11.51              |
|                 | 14   | TIP41| 1.484            | TIP41                | 2.338                           | 2.71    | 10.45              |
was GAPDH and SAND with a stability value of 0.046. The ranking of candidate reference genes and best combination of two genes from NormFinder were not identical to those from the geNorm analysis (Tables 2, 3 and 4).

**BestKeeper analysis.** Pfaffl proposed that genes with high SD or CV values can be considered inconsistent\(^1\). In general, a gene with an SD value greater than 1 is not acceptable (Tables 2, 3 and 4). For leaves of different developmental stages, GR was the optimal reference gene with an SD value of 0.30. For different tissues in the fruiting period and the group with glyphosate treatment, PP2Acs (SD values of 0.40 and 0.26, respectively) was the most stable gene. For the abiotic stress group, EF1\(\alpha\) (an SD value of 0.42) was the most stable of the candidate reference genes. For the hormone treatment and total samples group, BestKeeper ranked SAND as the best reference gene with SD values of 0.18 and 0.49, respectively.

**Table 4.** Gene expression stability ranked by four algorithms for the total samples group.

| Rank | Gene   | geNorm Stability | NormFinder Gene | NormFinder Stability | BestKeeper Gene | BestKeeper SD [± Cq] | BestKeeper CV (%Cq) | RefFinder Gene | RefFinder Stability |
|------|--------|------------------|----------------|----------------------|----------------|----------------------|---------------------|----------------|------------------|
| 1    | ACT    | 0.527            | EF1\(\alpha\)   | 0.275                | SAND           | 0.49                 | 2.07                | EF1\(\alpha\)   | 1.68             |
| 2    | PP2Acs | 0.527            | ACT             | 0.393                | EF1\(\alpha\)   | 0.51                 | 2.95                | ACT            | 1.86             |
| 3    | SAND   | 0.712            | SAND            | 0.412                | ACT            | 0.58                 | 3.16                | SAND           | 2.28             |
| 4    | EF1\(\alpha\) | 0.742       | RPL8            | 0.454                | PP2Acs         | 0.60                 | 2.87                | PP2Acs         | 3.36             |
| 5    | RPL8   | 0.810            | TUB             | 0.462                | GR             | 0.60                 | 2.43                | RPL8           | 5.14             |
| 6    | GR     | 0.853            | CYP             | 0.466                | TUB            | 0.69                 | 3.57                | GR             | 5.96             |
| 7    | CYP    | 0.879            | GR              | 0.470                | RPL8           | 0.70                 | 3.51                | TUB            | 6.62             |
| 8    | PP2Acs | 0.907            | EF1\(\alpha\)   | 0.482                | cIF            | 0.72                 | 3.43                | CYP            | 7.17             |
| 9    | GAPDH  | 0.937            | cIF             | 0.643                | CYP            | 0.73                 | 4.17                | cIF            | 8.97             |
| 10   | cIF    | 0.977            | GAPDH           | 0.654                | GAPDH          | 0.93                 | 5.08                | GAPDH          | 9.74             |
| 11   | UBQ    | 1.051            | UBQ             | 0.907                | UBQ            | 1.05                 | 5.60                | UBQ            | 11.00            |
| 12   | DNAI   | 1.179            | TIP41           | 1.564                | DNAI           | 1.24                 | 6.46                | DNAI           | 12.00            |
| 13   | TIP41  | 1.570            | DNAI            | 1.173                | TIP41          | 1.75                 | 7.57                | TIP41          | 13.00            |
| 14   | RUBP   | 1.644            | RUBP            | 2.162                | RUBP           | 1.83                 | 12.51               | RUBP           | 14.00            |

**Figure 2.** The relative expression patterns of P5CS under different treatments and with normalization to different reference genes. (a) Glyphosate treatment, (b) drought treatment, (c) salinity treatment, (d) heat treatment, (e) cold treatment, (f) ABA treatment, and (g) GA treatment. The error bars represent the standard error.
GIGANTEA (ΔCt) (Tables 2, 3 and 4). For leaves of different developmental stages and the glyphosate treatment group, the expression should increase or hold stable rather than decrease. Previous studies found that the expression of Arabidopsis thaliana and P5CS GI was resistant to herbicide20, indicating that lower expression of P5CS or GI indicates stronger herbicide tolerance.

As shown in Figs 2 and 3, the expression patterns of P5CS and GI were significantly different when using the best reference gene combination for normalization under different stress conditions than when normalized with the least stable reference gene. With the best gene combination, the P5CS level at 72 h was 2.2-fold higher than in the untreated sample; with the least stable reference gene, the expression at 72 h was 475-fold higher than in the untreated sample (Fig. 2). Using the stable gene combination, the expression level of P5CS was much higher than that produced by using the best gene combinations (see Supplementary Table S4). This indicates that the normalization with stable reference genes lowers the variability of the raw data, so the selected stable reference genes are acceptable. In our study, the expression normalized with the least stable reference genes DNAJ and TIP41 deviated dramatically from those normalized with the best gene combination. DNAJ, a

**Reference gene validation.** Delta 1-pyrroline-5-carboxylate synthetase (P5CS) is the rate-limiting enzyme in the proline synthesis pathway under stress conditions19. GIGANTEA (GI) participates in multiple molecular regulatory responses including flowering, circadian rhythm and stress tolerance19. The elevated expression of P5CS and GI improves resistance against salinity and drought stresses. Therefore, P5CS and GI expression should increase or hold stable rather than decrease. Previous studies found that the Arabidopsis thaliana gi mutant is resistant to herbicide20, indicating that lower expression of GI indicates stronger herbicide tolerance.

As shown in Figs 2 and 3, the expression patterns of P5CS and GI were significantly different when using the best reference gene combination for normalization under different stress conditions than when normalized with the least stable reference gene. With the best gene combination, the P5CS level at 72 h was 2.2-fold higher than in the untreated sample; with the least stable reference gene, the expression at 72 h was 475-fold higher than in the untreated sample (Fig. 2). Using the stable gene combination, the expression level of GI decreased significantly within 48 h of glyphosate treatment, which is consistent with the expected pattern; while using the worst reference gene, its expression dramatically increased (Fig. 3).

In the drought, salinity or abscisic acid (ABA) treatment groups, the expression of P5CS and GI increased or decreased slightly when using the best reference gene combinations for normalization, and the values ranged from 0.4 to 1.5; when using the least stable reference genes, these expressions decreased by 4 to 30-fold (Figs 2 and 3). The expression patterns of P5CS and GI normalized by stable reference gene combinations were more in line with the expectations than when they were normalized with the least stable reference genes. For the heat, cold and gibberellin (GA) stress groups, the expression of P5CS and GI ranged from 0.3 to 1.5 when normalized with the best reference gene combination; the expression normalized by the worst reference genes increased 3 to 40-fold (Figs 2 and 3).

For the relative expression of P5CS or GI, the coefficients of variation (CV (%)) using the poor reference genes were much higher than those produced by using the best gene combinations (see Supplementary Table S4). This indicates that the normalization with stable reference genes lowers the variability of the raw data, so the selected stable reference genes are acceptable. In our study, the expression normalized with the least stable reference genes DNAJ and TIP41 deviated dramatically from those normalized with the best gene combination. DNAJ, a
molecular chaperone, responds to stress and maintains protein homeostasis in plant cells. \[^1\] DNAJ rises sharply in response to stress, as observed in Figs 2 and 3. Similarly, TIP41 responds to abiotic stresses via the TOR signalling pathway. \[^2\] Under stress conditions, the acute decrease in TIP41 levels results in unreasonably higher normalized expression values (Figs 2 and 3).

### Discussion

Buffalobur (Solanum rostratum Dunal) is a worldwide noxious invasive weed, and it ranked as one of the top 10 alien invasive species in China. \[^1\] Many studies have focused on understanding the physiological characteristics of this weed, including seed germination and seedling emergence. \[^3\] For genetic research, it is necessary to select stable reference genes to ensure the accuracy of research results. In this study, three software packages and one web tool were used to test the statistical reliability of candidate reference genes.

Notably, the different algorithms evaluating the expression stability of reference genes generated different stable genes due to their different mathematical calculations. One disadvantage of geNorm is that it is likely to select coregulated genes with similar expression profiles, such as genes in the same functional class. When groups are specified, NormFinder considers the inter- and intragroup variations for normalization factor calculation and eliminates artificial selection of coregulated gene values; however, it cannot exclude systematic errors generated from sample preparation. \[^4\] For BestKeeper, input data derived from more than three candidate genes are required for accurate assessment of the stability of each gene. \[^5\] Based on different calculation principles, the stability rankings of three statistical packages were different. RefFinder considers four statistical approaches (geNorm, NormFinder, BestKeeper and Delta-Ct); therefore, we used RefFinder to obtain a comprehensive ranking of gene expression stability.

In our study, the combination of EF1α, ACT (β-actin) and SAND was sufficient to normalize expression levels of target genes in the total samples group. Therefore, it is recommended that EF1α, ACT and SAND should be used together for normalization in various buffalobur experimental samples. ACT, which is a cytoskeleton component and cell division regulator, is the most stable gene in leaves of different developmental stages and glyphosate-treated leaves (Tables 2 and 3). ACT is also stably expressed in Hordeum vulgare L. \[^6\] In Descurainia sophia, actin 7 (ACT7) is suitable in most samples under different conditions, and actin 8 (ACT8) is the least stable reference gene. \[^7\] SAND is the most stable reference gene for different tissues in the fruiting period and in hormone-stimulated leaves (Tables 2 and 3). Similarly, SAND is stable in different tomato tissues. \[^8\] In Stellera chamaejasme and Robinia pseudacacia L., SAND levels are stable under ABA and drought treatments, respectively. \[^9\] In Paeonia lactiflora Dunn, SAND and actin 2 (ACT2) are the top two most stable reference genes under abiotic stress and hormone treatments and in different tissues. \[^10\] EF1α, which participates in the elongation cycle of protein biosynthesis, is the most stable gene for leaves under abiotic stress or hormone stimulation (Tables 2 and 3). In potato, the expression of EF1α rises sharply in response to stress, as observed in Figs 2 and 3. Similarly, TIP41 responds to abiotic stresses via the TOR signalling pathway. \[^11\] Under stress conditions, the acute decrease in TIP41 levels results in unreasonably higher normalized expression values (Figs 2 and 3).
developmental stages in tomato; however, in buffalobur, it ranks behind other reference genes in different tissues of the fruiting period and leaves of different developmental stages. It showed that reference genes used in tomato might not be suitable in genetically related species. Our results here further emphasize the importance of species-specific screening of proper reference genes because genetic difference, even in closely related species, may contribute to distinct and variable expression of these genes under the same experimental conditions.

P5CS is stress-responsive as it is involved in the synthesis of key enzymes in the proline synthesis pathway. GI participates in developmental processes such as plant flowering, but it is also involved in mediating cold stress and salt stress responses. Under stress conditions such as salinity, drought and ABA, the expression of P5CS and GI are expected to increase or hold stable rather than decrease to help plants adapt to the negative conditions. In our research, the P5CS or GI expression levels normalized using the best reference gene combination were significantly different from those normalized with the least stable reference gene (Figs. 2 and 3). With the best gene combination for normalization, the expression of P5CS and GI were only slightly different before and after stresses; with the least stable reference gene, P5CS and GI expression rose or fell sharply (Figs 2 and 3). Therefore, the reference genes selected in this study are reliable.

In summary, 14 candidate reference genes were first selected under different treatments in buffalobur. For leaves of different developmental stages and leaves of glyphosate-treated plants, the best reference genes are ACT and EF1α. For different tissues in the fruiting period, the most stable gene pair is SAND and PP2Acs. EF1α and RPL8 are the most stable reference genes for the abiotic stress group. EF1α and SAND are suitable reference genes for the hormone stimuli group. For the total samples group, the EF1α, ACT and SAND triplet should be used as reference genes for normalization. This study will facilitate the study of gene expression analysis in buffalobur, which might lay a fundamental path for exploring the molecular mechanism underlying its developmental regulation and for effectively controlling this invasive weed species.

Materials and Methods

Plant materials and stress treatments. Buffalobur seeds were collected from the Miyun District (N40.24.082, E116.50.364), Beijing, China, in 2017. Seeds were sown in pots (11 cm diameter) filled with Pindstrup substrate (SIA Pindstrup, Balozi, Latvia) and grown in chambers under a 14 h light/10 h dark photoperiod with 300 μmol⋅m−2⋅s−1 of light intensity at 30 ± 2°C. When the seedlings were at the 3-leaf stage, they were subjected to three types of stress treatments including abiotic stress (drought, salinity, cold, and heat), hormone stimuli (ABA and GA) and herbicide treatment (glyphosate) (Table 5). For salt or drought treatments, approximately 150 mL of NaCl (300 mM) or 20% PEG-6000 was applied to irrigate the plants; for cold or heat treatments, the plants were transferred to chambers at a temperature of 4°C or 40°C under the same photoperiod and light intensity as previously specified; for hormone treatment, the leaves were sprayed with 0.35 mM ABA (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) or 0.35 mM GA (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China); and for glyphosate treatment, 1680 g ae ha−1 of glyphosate (Roundup, isopropylamine salt of glyphosate, 410 g ae L−1, Monsanto Company, St. Louis, USA) was sprayed on the leaves. The fifth leaves were collected at 0 (untreated), 24, 48 and 72 h after treatment. Three independent biological replicates per treatment were collected, immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Developmental tissue samples. Buffalobur seeds were sown in pots and grown in chambers under the conditions mentioned above. The leaves were collected at different developmental stages; the collected leaves included the cotyledons (cotyledon stage) and the fully expanded 3rd (seedling stage), 5th (vegetative stage) and 7th (fruiting period) leaves (Table 8).

For different tissues in the fruiting period, the roots, stems (6 cm above the root), 7th leaves, petals (without stamens and pistils), and pericarps (with thorns) were collected (Table 5). Three independent biological replicates per treatment were collected, and the storage procedure was same as that for the stress-treatment samples.

RNA extraction and cDNA synthesis. Total RNA isolation was conducted using the common method described by Chen et al.35. The concentration and purity of total RNA were quantified using the NanoDrop™ One/OneC ultra trace UV spectrophotometer (ThermoFisher, Waltham, MA, USA). The A260/A280 values of the RNA samples ranged from 1.90 to 2.10. The RNA integrity was assessed using 1% agarose electrophoresis gels stained with Solargel Red nucleic-acid dyes (Solarbio, Beijing, China), and gels of all RNA samples exhibited sharp and intense bands for 18S (see Supplementary Fig. S5). cDNA synthesis was performed with 800 ng of total RNA in a final volume of 20 μL using the same kit described by Chen et al., and the synthesized cDNA was stored at −80°C until use.

Primer design and qPCR assay. Fourteen candidate reference genes (GAPDH, ACT, GR, UBQ, TIP41, RPL8, eIF, TUB, DNAJ, CYP, EF1α, PP2Acs, RUBP, and SAND) were selected for the stress expression assay, and the GenBank accession numbers of these genes are MG930815, MG930814, MG930816, MG930817, KT807935, KT807936, KT596731, KT807934, KT596730, MK181638, MK181640, MK181641, MK181642, respectively. The primers for qPCR were designed using the Oligo 7 software; information on the primers is presented in Table 1. Polymerase chain reaction (PCR) was performed to confirm the 14 candidate reference gene sequences, and the primers amplified a single correct target product without visible primer dimers (see Supplementary Fig. S6). The qPCR reactions were performed using the 7500 RealTime PCR System (Applied Biosystems, Waltham, MA, USA) for thermal cycling and SYBR Green detection chemistry (Applied Biosystems, CA, USA). The reaction mixtures and cycling conditions were based on the method described by Chen et al.35.

A single peak was detected on the melting curve for each primer pair after qPCR, which further demonstrated the specificity of these primers (see Supplementary Fig. S7). To confirm the specificity of the primer pairs, the
amplification efficiency (E) and correlation coefficient (R²) parameters were obtained from the standard curves using the common method. The amplification efficiencies of 14 candidate reference genes ranged from 90.0% to 104.7%, and the correlation coefficients (R²) were between 0.990 and 0.998 (Table 1; see Supplementary Fig. S8).

Data analysis for expression stability. We chose three statistical software programs (geNorm, NormFinder, and BestKeeper) and a web tool (RefFinder) to evaluate the stability of 14 candidate reference genes. The analysis methods of these four programs were the same as those reported in other published articles. geNorm identifies the best reference gene pair by calculating the value M. The smaller the M value, the more stable the gene expression. Furthermore, geNorm also determines the optimal number of reference genes needed by calculating the pairwise variation (Vn/Vn+1). NormFinder evaluates gene expression stability via grouped analysis. The output is not simply the best gene but also the best combination of two genes. The value of expression stabilities derived from NormFinder is smaller, and the single reference gene is more stable. BestKeeper employs pair-wise correlation analysis of all pairs of candidate reference genes to estimate gene expression stability. Pfaffl considers genes with elevated SD or CV values inconsistent. RefFinder generates a comprehensive ranking synthesized using the results of four algorithms (geNorm, NormFinder, BestKeeper and Delta-Ct).

Validation of reference genes. To validate the reliability of the obtained reference genes, the expression of two target genes—delta 1-pyrroline-5-carboxylate synthetase (P5CS) and GIGANTEA (GI)—under different experimental conditions was normalized using the combination of the two best reference genes and the most variable gene obtained via RefFinder (see Supplementary Tables S9, S10 and S11). The GenBank accession numbers of P5CS and GI are MK181643 and MK181644, respectively. Information on the primers for these two target genes is listed in Table 1. The expression levels of these two target genes normalized to the reference genes were analysed using the 2−ΔΔ Ct method. We set up three biological and technical replicates in qPCR assays.

Data Availability. The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions
S.H.W., H.J.H. and D.D.Z. conceived and designed research. D.D.Z., J.C.C. and X.W. conducted experiments. Z.F.H. and C.L.J. contributed reagents and analytical tools. D.D.Z. and X.W. analyzed data. D.D.Z., J.C.C., C.X.Z. and H.Q.H. wrote the manuscript.

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