Reference gene selection for quantitative real-time PCR (qRT-PCR) expression analysis in *Galium aparine* L.

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Abstract

To accurately evaluate expression levels of target genes, stable internal reference genes is required for normalization of quantitative real-time PCR (qRT-PCR) data. However, there have been no systematical investigation on the stability of reference genes used in the bed-straw weed, *Galium aparine* L. (BGA). In this study, the expression profiles of seven traditionally used reference genes, namely 18S, 28S, ACT, GAPDH, EF1α, RPL7 and TBP in BGA were assessed under both biotic (developmental time and tissue), and abiotic (temperature, regions and herbicide) conditions. Four analytical algorithms (geNorm, Normfinder, BestKeeper and the ΔCt method) were used to analyze the suitability of these genes as internal reference genes. RefFinder, a comprehensive analytical software, was used to rank the overall stability of the candidate genes. The optimal normalization internal control genes were ranked as: 28S and RPL7 were best for all the different experimental conditions (developmental stages, tissues, temperature, regions and herbicide treatment); 28S and RPL7 for developmental stages; TBP and GAPDH for different tissues; 28S and GAPDH were relatively stable for different temperature; 28S and TBP were suitable for herbicide treatment. A specific set of reference genes were recommended for each experimental condition in BGA.

Introduction

Quantitative Real-time PCR (qRT-PCR) is considered as the most common method for gene expression quantification, due to high sensitivity and efficiency[1–3]. However, the accuracy of qRT-PCR can be influenced by the quality of the template, the efficiency of transcription and amplification, and experimental procedures between samples[4]. To enhance the accuracy of qRT-PCR analysis, several strategies such as normalization of sample size, ensurence of the RNA quality and quantity, and removal of DNA contamination were carried out [5]. Among these strategies, normalization of gene expression is essential to prevent nonbiological alteration or incorrection, and the currently preferred practice is to determine the target gene expression with an internal reference gene as control[1, 6]. No reference gene exhibits stable
expression under all conditions. Therefore, it is essential to select stable reference genes for determining the transcript changes of target gene by qRT-PCR. Besides, it is integral to estimate suitable reference genes for each specific condition. Several algorithms, such as geNorm [7], BestKeeper [8], and NormFinder [9] were used for candidate reference genes evaluation. The frequently used housekeeping genes, such as β-actin (ACT), α-tubulin (TUA), ubiquitin (UBQ), cyclophilin (CYP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S or 28S ribosomal RNA and elongation factors (EF) have been verified to be suitable as internal control genes in many plants [10–12].

Bedstraw weed, Galium aparine L. (BGA), a malignant weed from the Rubiaceae family, can cause great harm to oilseed rape, wheat and some other crops in China [13]. The control of this weed normally relies on chemical herbicides for decades. However, the wide, unreasonable and dominant application of chemical herbicides not only caused cropland deterioration, but also caused rapid development of weed resistance to chemical herbicides [14]. Resistance mechanisms usually included target-site resistance (TSR) and/or non-target site resistance (NTSR) mechanisms. Both the over-expression of target proteins and structural changes to the herbicide-binding sites belonged to TSR mechanisms. Hence, it was necessary to select the suitable reference genes for evaluation the target gene expression in weed species. To date, the expression levels of reference genes have been commonly quantified in various weeds by qRT-PCR [15,16]. Only one reference gene such as 18S, GAPDH, or ACT was selected for normalization the target gene expression by qRT-PCR under all the diverse experimental conditions in multiple studies. However, it is indispensable to use at least 2 or 3 reference genes for achieving accurate normalization [7,17].

Reference gene validation in weeds is lacking of systematization and should be paid more attention, especially in agricultural weeds. Hence, it is important to assess stable reference genes in BGA for qRT-PCR quantification. Seven common reference genes, such as 18S, 28S, ACT, GAPDH, EF1α, RPL7 and TBP, were selected and evaluated for target gene expression normalization using the qRT-PCR method in BGA. Our results revealed that specific reference genes were required for qRT-PCR normalization under various experimental conditions.

Materials and methods

Cultivation of seedlings

The seeds of BGA used in this study were collected from a Xinxiang county wheat field (35°18′13.71″N, E113°55′15.05″E) in Henan Province, China, in Year 2010. The wheat field belonged to our college and collection was permitted by our college. Cultivation of BGA was adopted by the greenhouse potting methods [18]. The seeds of BGA were sown into 75 cm² pots filled with a mixture of soil sand and grass biochar, and germinated in the greenhouse under 20 / 15 °C day/night temperatures with a 12 / 12 h (light /dark) cycle and 75 ± 5% relative humidity.

Biotic conditions. BGA foliar parts were obtained with specific developmental stage including 1-leaf stage, 2-leaves stage, and 3-leaves stage. Different vegetative tissue including leaves, roots, and stems were seperately collected. Ten individual plants was randomly selected per repetition and three biological replicates were used in this study. After collection, all samples were rapidly frozen in liquid nitrogen and stored at -80 °C for subsequent total RNA isolation and qRT-PCR testing.

Abiotic conditions

To examine the effect of herbicide, the concentration of herbicide tribenuron-methly was determined as 10 g ai/ha (IC₁₀) by a modified whole-plant assay [19]. 3-leaves stage BGA plants
with height about 20 cm were exposed to tribenuron-methly at the corresponding IC<sub>10</sub> concentration with an auto spray device (ASP-1098) under 0.2 MPa pressure. The liquid volume was 450 L each ha. Water alone was selected as control. Foliar parts were collected after 24 h treatment, quickly frozen with liquid nitrogen before storage at −80 °C.

**Selection of reference gene and primer design**

To identify suitable reference genes for qRT-PCR analysis in BGA, seven common reference genes, such as 18S ribosomal RNA (18S rRNA), 28S rRNA (28S), actin (ACT), elongation factor 1 alpha (EF1α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L7 (RPL7), and TATA box binding protein-associated factor (TBP), were selected for qRT-PCR analysis. The primers used for qRT-PCR were designed with the Primer3.0 software. The corresponding primer information were listed in Table 1. Besides, the detailed sequences of the seven selected reference genes were shown in S1 File.

**Total RNA extraction and cDNA synthesis**

Total RNA was isolated using TRIZol (Invitrogen, USA) according to the manufacturer’s recommendations. The RNA integrity was determined by electrophoresis with 1% agarose gel. The quality and quantity of RNA were measured using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., USA). Each sample of 1 μg RNA was reverse transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Japan) following the manufacturer’s instructions in a total volume of 20 μl. The cDNA templates were stored at -20˚C until use in qRT-PCR.

**Quantitative real-time PCR (qRT-PCR)**

qRT-PCR was implemented in a 7500 Real-Time PCR system (Applied Biosystems, USA). Each reaction (20 μl) contained 1 μl of cDNA template, 10 μl of SYBR Green qRT-PCR Table 1. Primer information of candidate reference genes and a target gene.

| Gene Symbol | Gene name                      | Tm (˚C) | Sequence (5´–3´)                  | Product Length (bp) |
|-------------|--------------------------------|---------|----------------------------------|---------------------|
| ACT         | Actin                          | 57.45   | F: GGACCGGTTTGAGTTCGAGAA         | 111                 |
|             |                                | 55.40   | R: ACTACGGCCAACTTTGTCAA          |                     |
| 18S         | 18s ribosomal                  | 57.45   | F: GCAGTCTCCATCAACCACCT          | 106                 |
|             |                                | 57.45   | R: CTTCACCAGCTAACATCCAG          |                     |
| 28S         | 28s ribosomal                  | 57.45   | F: TTGTCCGGCATAAACCTGGG          | 98                  |
|             |                                | 55.40   | R: AAGGACTATTTCCGCGACTCT         |                     |
| GAPDH       | Glyceraldehyde-3-phosphate     | 55.40   | F: GCCCATTTTCAGCTGCAA            | 117                 |
|             |                                | 55.40   | R: TTTGCAGCCAATCTTTCC            |                     |
| EF1α        | Elongation factor 1 alpha      | 57.45   | F: TGTGACGCTCCCTTGAGATG          | 93                  |
|             |                                | 57.45   | R: TAGTCACGGCAAACCTCTCAG         |                     |
| TBP         | TATA box binding protein       | 57.45   | F: TTGGGTGTACGTGAGAGGC          | 112                 |
|             |                                | 57.45   | R: CAGTGGCGTGAATGAGAGGA         |                     |
| RPL7        | Ribosomal protein L7           | 55.40   | F: AAGACGGAAGAGCTGCAGA          | 106                 |
|             |                                | 55.40   | R: CAACCTTTCTATGCCCCGAG         |                     |
| HSP70       | Heat shock protein 70         | 58.98   | F: ACCCTGCACCTGAGTGTTA          | 105                 |
|             |                                | 58.93   | R: GGCTGCTCCTTCTTCTTCT           |                     |

Notes: F, forward primer; R, reverse primer; Tm, melting temperature; R<sup>2</sup>, coefficient of determination

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SuperMix-UDG (Invitrogen, USA), 0.3 μl of each specific primer, and 8.7 μl of nuclease-free water. No cDNA template but corresponding volume nuclease-free water was used as NTC.

The amplification conditions for qRT-PCR were set as follows: 50˚C for 2 min, 95˚C for 2 min, and 40 cycles of 95˚C for 15 s, and 55˚C for 30 s. For melting curve analysis, a dissociation step cycle (from 65 to 95˚C) was carried out to verify the gene-specific amplification. Relative standard curves for the transcripts were conducted with a 5-fold dilution series of cDNA. The corresponding qRT-PCR efficiencies (E) were calculated using the equation: \[ E = \left(10^{-1/slope} - 1\right) \times 100\% \] [20–22]. All operations were processed with three biological replications and at least two technical replications.

Constancy analysis of reference gene expression

The stability of the seven candidate reference genes was assessed using the comparative ΔCt method [23], geNorm [7], NormFinder [9], and BestKeeper [8]. RefFinder, one free available online software was also applied to further estimate the stability of reference genes with the advantage of integrating the above all four major computational programs.

The geNorm program provides a calculation of gene expression stability value (M), and the reference gene with the lowest M value is considered as the most stable gene. In general, the candidate reference gene is considered stable when an M value below 1.5. The geNorm also performs a pairwise variation (V) with a serial ratio of \[ V_{n/n+1} \]. Addition of another reference gene could be required for accurate normalization when a \[ V_{n/n+1} \] value above 0.15. NormFinder software calculates the expression stability of the candidate reference gene in all given sample set and ranks the stability order of candidate genes [9]. BestKeeper considers the Ct values of all the selected reference genes to determine the standard deviation for screening the “optimal” reference genes. The ΔCt method directly make a relative expression comparison between two candidate genes within the same sample. RefFinder combines the above four methods and gives the overall final ranking order.

Validation of selected reference gene

One heat shock gene (HSP70) was regarded as the target gene to validate the reference genes stability. HSP70 expression levels under diverse factors were assessed according to different candidate reference genes as the internal control genes. Normalization factors (NFs) were computed based on the geometric mean values which was determined by RefFinder. The expression profiles of HSP70 in different treatment were conducted with the \[ 2^{-\Delta\Delta C T} \] method [20].

Statistical analysis

Statistical analysis were performed with the software InStat v.3.0 (GraphPad Software, San Diego, CA). One-way ANOVA test was chosen for different comparison of target gene expression with significance \( P < 0.05 \).

Results and discussion

Evaluation of reference genes expression stability

All the primer pairs of seven selected candidate reference genes and one target gene (HSP70) was specific through evaluation by PCR. For each candidate reference gene, a single PCR product band with the expected size was visualized by 1.5% agarose gel. Besides, gene-unique amplification was also corroborated by a single qRT-PCR melting curve peak. Standard curve analysis revealed that the amplification efficiency of qRT-PCR varied from 90.5 to 105.3%
Besides, the linear regression correlation coefficients ($R^2$) of all the 8 genes ranged from 0.983 to 0.998 (Table 1).

The mean Ct values of all the tested reference genes were between 27.24 (ACT) to 31.21 (18S), covering all the experimental conditions (Fig 1). Among the candidate genes, ACT was the most accumulated transcripts, indicated by the lowest mean Ct value of 27.24, whereas 18S had the lowest transcript expression level with a mean Ct value of 31.21.

For tissue samples, the rankings of reference gene stability were similar based on $\Delta$Ct method and Normfinder, showing TBP and RPL7 as the top two most stable genes. While, TBP was considered as the most stable gene using BestKeeper and geNorm (Table 2). RefFinder produced the stability rankings from most stable to least as TBP, GAPDH, 28S, RPL7, 18S, EF1α and ACT (Fig 2A). GeNorm indicated that the gene number would be suitable for normalization. In comparison with the cut-off value of 0.15, the $V_{3/4}$, $V_{5/6}$, and $V_{6/7}$ values were exceeded (Fig 3), indicating that additional reference genes are required.

For different developmental stages, the stability rankings were almost the same using $\Delta$Ct method and NormFinder with TBP and EF1α as the top two most stable candidate reference genes. GeNorm analysis identified 28S and EF1α as the most and the least stable reference genes, respectively. GAPDH and EF1α were demonstrated as the top two most stable reference genes by Bestkeeper (Table 2). Integrating the results from all four programs, RefFinder
Table 2. Expression stability of the candidate reference genes in response to different conditions.

| Conditions          | ΔCt Stability | Rank | BestKeeper Stability | Rank | Normfinder Stability | Rank | geNorm Stability | Rank |
|---------------------|---------------|------|----------------------|------|----------------------|------|------------------|------|
| Different tissues   |               |      |                      |      |                      |      |                  |      |
| ACT                 | 2.455         | 7    | 2.462                | 7    | 2.138                | 7    | 1.946            | 7    |
| 18S                 | 2.185         | 5    | 1.288                | 5    | 1.776                | 5    | 1.426            | 5    |
| 28S                 | 1.942         | 4    | 0.636                | 3    | 1.469                | 4    | 1.023            | 3    |
| GAPDH               | 1.705         | 3    | 0.565                | 2    | 1.081                | 3    | 0.696            | 1    |
| EF1α                | 2.253         | 6    | 2.068                | 6    | 1.840                | 6    | 1.745            | 6    |
| TBP                 | 1.525         | 1    | 0.403                | 1    | 0.644                | 2    | 0.696            | 1    |
| RPL7                | 1.570         | 2    | 1.181                | 4    | 0.352                | 1    | 1.148            | 3    |
| Developmental sages |               |      |                      |      |                      |      |                  |      |
| ACT                 | 2.183         | 7    | 1.402                | 7    | 2.085                | 7    | 1.429            | 7    |
| 18S                 | 1.420         | 5    | 1.170                | 6    | 1.058                | 5    | 0.858            | 3    |
| 28S                 | 1.250         | 3    | 0.758                | 4    | 0.763                | 4    | 0.604            | 1    |
| GAPDH               | 1.325         | 4    | 0.397                | 1    | 0.759                | 3    | 1.128            | 6    |
| EF1α                | 1.215         | 2    | 0.538                | 2    | 0.435                | 1    | 1.049            | 5    |
| TBP                 | 1.173         | 1    | 0.725                | 3    | 0.570                | 2    | 0.738            | 2    |
| RPL7                | 1.438         | 6    | 1.091                | 5    | 1.185                | 6    | 0.604            | 1    |
| Herbicide treatments|               |      |                      |      |                      |      |                  |      |
| ACT                 | 2.234         | 6    | 1.235                | 4    | 1.224                | 5    | 1.022            | 6    |
| 18S                 | 2.226         | 5    | 1.641                | 6    | 1.656                | 6    | 0.864            | 5    |
| 28S                 | 1.701         | 2    | 0.863                | 2    | 0.075                | 1    | 0.150            | 1    |
| GAPDH               | 1.990         | 4    | 0.610                | 1    | 0.376                | 3    | 0.723            | 3    |
| EF1α                | 6.638         | 7    | 4.652                | 7    | 6.605                | 7    | 2.626            | 7    |
| TBP                 | 1.691         | 1    | 0.867                | 3    | 0.075                | 2    | 0.150            | 1    |
| RPL7                | 1.904         | 3    | 1.383                | 5    | 1.055                | 4    | 0.513            | 2    |
| Temperature         |               |      |                      |      |                      |      |                  |      |
| ACT                 | 2.660         | 7    | 2.284                | 7    | 2.476                | 7    | 1.779            | 7    |
| 18S                 | 1.406         | 1    | 0.906                | 2    | 0.529                | 1    | 0.986            | 3    |
| 28S                 | 1.432         | 2    | 1.480                | 5    | 0.566                | 2    | 0.460            | 1    |
| GAPDH               | 1.560         | 4    | 1.529                | 6    | 0.978                | 4    | 0.460            | 1    |
| EF1α                | 2.041         | 6    | 0.701                | 4    | 1.657                | 6    | 1.427            | 6    |
| TBP                 | 1.522         | 3    | 1.299                | 4    | 0.886                | 3    | 0.791            | 2    |
| RPL7                | 1.836         | 5    | 0.931                | 3    | 1.453                | 5    | 1.210            | 5    |
| Region              |               |      |                      |      |                      |      |                  |      |
| ACT                 | 1.476         | 5    | 1.410                | 7    | 1.123                | 5    | 1.084            | 4    |
| 18S                 | 1.338         | 4    | 1.133                | 5    | 1.011                | 4    | 0.633            | 1    |
| 28S                 | 1.250         | 3    | 1.165                | 6    | 0.837                | 3    | 0.701            | 2    |
| GAPDH               | 1.740         | 7    | 0.977                | 4    | 1.498                | 7    | 1.374            | 7    |
| EF1α                | 1.494         | 6    | 0.869                | 1    | 1.132                | 6    | 1.228            | 6    |
| TBP                 | 1.145         | 1    | 0.876                | 2    | 0.622                | 2    | 0.633            | 1    |
| RPL7                | 1.176         | 2    | 0.912                | 3    | 0.563                | 1    | 0.830            | 3    |
| Pooled samples      |               |      |                      |      |                      |      |                  |      |
| ACT                 | 2.941         | 6    | 2.033                | 6    | 2.611                | 6    | 1.585            | 6    |
| 18S                 | 1.946         | 2    | 1.316                | 3    | 1.313                | 2    | 0.864            | 3    |
| 28S                 | 2.382         | 4    | 1.910                | 5    | 1.985                | 4    | 1.126            | 4    |
| GAPDH               | 1.952         | 3    | 1.150                | 2    | 1.356                | 3    | 0.661            | 2    |
| EF1α                | 3.523         | 7    | 1.729                | 4    | 3.286                | 7    | 1.883            | 7    |
| TBP                 | 1.689         | 1    | 1.129                | 1    | 0.889                | 1    | 0.389            | 1    |
| RPL7                | 2.543         | 5    | 2.115                | 7    | 2.194                | 5    | 1.329            | 5    |

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provided a ranking as 28S, RPL7, TBP, 18S, EF1α, GAPDH and ACT (Fig 2B) across different developmental stages. In comparison with the cut-off value of 0.15, the pair-wise value of V_{6/7} was above 0.15 (Fig 3), suggesting the number of reference genes should be added for accurate normalization.
For three different regions, the top two most stable reference genes were identified as TBP and RPL7 by the ΔCt method and NormFinder, respectively. Analysis using BestKeeper showed that the most stable reference gene for normalization was EF1α. According to RefFinder, the ranking of seven selected reference genes from the highest to lowest stability was: TBP, 18S, 28S, RPL7, ACT, EF1α, and GAPDH. GeNorm showed that all the pair-wise values of $V_{n/n+1}$ were below 0.15 across different regions including Xinxiang, Kaifeng, and Luohe (Fig 3).

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For different temperatures, the ΔCt method and NormFinder results exhibited that 18S and ACT were considered as the most stable and the least reference gene, respectively. GeNorm results revealed that 28S and GAPDH were the top two most stable reference genes. The expression stability of the seven reference genes from the most to the least stable was comprehensively ranked as 28S, GAPDH, TBP, 18S, RPL7, EF1α and ACT based on RefFinder (Fig 2C). From geNorm analysis, the \(V_{n/n+1}\) values were all below 0.15 across different temperatures (Fig 3), indicating no supplemental reference gene was added for normalization of the qRT-PCR analyses. Thus, 28S and GAPDH were the most suitable candidate reference genes across different temperatures.

For three different regions, the top two most stable reference genes were identified as TBP and RPL7 by the ΔCt method and NormFinder, respectively. Analysis using BestKeeper showed that the most stable reference gene for normalization was EF1α. According to RefFinder, the ranking of seven selected reference genes from the highest to lowest stability) was: TBP, 18S, 28S, RPL7, ACT, EF1α, and GAPDH (Fig 2D). GeNorm showed that all the pair-wise values of \(V_{n/n+1}\) were below 0.15 across different regions including Xinxiang, Kaifeng, and Luohe (Fig 3).

For herbicide application, the most stable reference gene was TBP based on ΔCt method and geNorm. TBP and 28S were the top two ranked genes by geNorm (Table 2). However, EF1α was the least stably expressed reference gene by employing all the four different algorithms. Based on RefFinder, the order of reference gene stability ranking across herbicide application was: 28S, TBP, RPL7, GAPDH, 18S, ACT and EF1α (Fig 2E). GeNorm showed that the \(V_{n/n+1}\) values were below 0.15 across different regions including Xinxiang, Kaifeng, and Luohe (Fig 3).

For all the experimental conditions, the ranking order of reference gene stability was the same according to Ct method and NormFinder, and TBP and EF1α were confirmed as the most stable and the least stable one, respectively. Furthermore, ACT was confirmed as the less unstable reference gene by all of them (Table 2). Based on RefFinder, the stability ranking order of reference gene across various conditions was: TBP, GAPDH, 18S, 28S, RPL7, ACT, and EF1α (Fig 2F). All pairwise variations with the \(V_{n/n+1}\) values were evaluated using GeNorm across diverse conditions (Fig 3). Therefore, 28S and RPL7 were determined to be the best housekeeping genes under various conditions.

qRT-PCR has been broadly used for quantification of the gene transcript abundance. The accurate and reliable qRT-PCR results were always affected by diverse factors including RNA quality, cDNA synthesis, experimental procedures, and reference gene normalization. Therefore, reliable reference gene selection is of particular importance for successful qRT-PCR analysis. To date, no study have systematically been focused on evaluation of the reference gene stability in BGA.

Our results indicated that 28S and RPL7 were the top two most stable reference genes across various experimental conditions according to RefFinder analysis and four different algorithms including ΔCt method, geNorm, BestKeeper, and NormFinder. The ranking order of reference gene stability can vary due to different algorithms with the four analytical tools. With the integration the four statistical methods, RefFinder, a comprehensive web-based tool, was chosen to rank the overall stability of the seven candidate reference genes. In this study, 28S and RPL7 were considered as the top two most stable reference genes using RefFinder within specific condition. However, GAPDH, ACT and EF1α were identified as the unstable reference genes across development stages, tissues, temperatures, regions, and herbicide treatments, sexes. Similarly, 18S rRNA or 28S was identified as the most stable reference gene in rice[24], the Australian sheep blowfly[25], and eggplant[26]. In contrast, GAPDH was reported as one of
the most stably expressed genes in weed species *Lolium* sp.[27] and *Avena fatua*[28]. GAPDH was also identified unstable in *Petunia hybrida* across development stage[29]. EF1α was considered to be the reliable reference gene under given experimental conditions in current study, and this is the same as results of some insects such as bumblebee[30] and cotton bollworm [31].

Liu et al.[28] evaluated TBP as the best reference gene in *Avena fatua*, and TBP was also identified as an ideal reference gene in tomato [32] and annual ryegrass[33]. Our results demonstrated that traditional reference gene ACT, involved in cytoskeleton structure, was not usually unchange under diverse experimental treatments. Similar findings were verified in the beetle *Tribolium castanenum*[34], perigord black truffle[35] and *Avena fatua*[36]. Therefore, no reference genes in multiple species show unchanged expression across all the tested conditions [37,38], suggesting that it is of particular important to identify suitable reference genes in BGA.

**Validation of selected reference genes**

To validate the recommended reference genes in this study, the relative expression level of one target gene, HSP70, was analyzed under all the experimental conditions. For different regions of BGA, the relative expression level of HSP70 was significantly up-regulated in Luohe and Kaifeng than in Xinxiang when using the most stable reference gene (NF1: TBP), the recommended normalization combination (NF1–2: TBP and 18S), and the least stable reference gene (NF7: GAPDH), significant difference was found in HSP70 expression when using single reference gene (the most stable, TBP) or reference gene combination (NF1–2: TBP and 18S) and single reference gene (the least stable, GAPDH) (Fig 4A).

For BGA exposed to different temperatures, the relative expression level of HSP70 expression was significantly higher at 25˚C or 35˚C than at 15˚C. The relative expression levels of HSP70 increased less at 35˚C using the least stable reference gene (28S) and the recommended normalization factors (GAPDH and 28S) as normalization (Fig 4B). Normalized by stable reference gene, the combination of the two most stable reference genes or the least stable reference gene, significant difference was found in HSP70 expression when treated with herbicide when using single reference gene (the most 28S) or reference gene combination (28S and TBP) and and single reference gene (the least EF1α). Meanwhile, HSP70 was significantly up-regulated under the herbicide stress in comparison with the control groups. The expression level of HSP70 was significantly increased by normalization with the least stable reference gene (28S) than by the most stable reference gene (GAPDH) and the recommended combination (ACT and GAPDH) (Fig 4C).
HSP70, an important stress-inducible heat shock protein gene, is as a target gene to validate its relative expression using the most stably expressed reference gene, the most unstably expressed one, and the recommended combination. The qRT-PCR analyzed results indicated that the transcriptional levels of target gene HSP70 varied significantly with normalization of different reference gene. This study represents the first attempt to select a set of candidate reference genes in BGA under various conditions for the normalization of gene expression data using qRT-PCR. 28S and RPL7 were identified as the most stably expressed reference genes under all experimental conditions, which provided a standardized procedure for the quantification of gene expression in BGA.

Supporting information
S1 File. Sequences of reference and target genes tested in *Galium aparine.* (XLS)

Author Contributions
Conceptualization: Xiling Chen.
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