

## Selection and evaluation of potential reference genes for gene expression analysis in *Avena fatua*

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**Citation:** Liu J., Li P., Lu L., Xie L., Chen X., Zhang B. (2019): Selection and evaluation of potential reference genes for gene expression analysis in *Avena fatua*. *Plant Protect. Sci.*, 55: 61–71.

**Abstract:** Eight commonly used candidate reference genes, 18S ribosomal RNA (rRNA) (*18S*), 28S rRNA (*28S*), actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor 1 alpha (*EF1α*), ribosomal protein L7 (*RPL7*), Alpha-tubulin (*α-TUB*), and TATA box binding protein-associated factor (*TBP*), were evaluated under various experimental conditions to assess their suitability in different developmental stages, tissues and herbicide treatments in *Avena fatua*. The results indicated the most suitable reference genes for the different experimental conditions. For developmental stages, *28S* and *EF1α* were the optimal reference genes, both *EF1α* and *28S* were suitable for experiments of different tissues, whereas for herbicide treatments, *GAPDH* and *ACT* were suitable for normalizations of expression data. In addition, *GAPDH* and *EF1α* were the suitable reference genes.

**Keywords:** wild oat; expression; herbicide

The internal control of target gene measurement refers to the use of reference gene expression variation and is the currently preferred method for normalising quantitative real-time reverse transcription polymerase chain reaction (qPCR) data because reference genes can capture all nonbiological variations (LOGAN *et al.* 2009). Although no gene exhibits constant expression under all experimental conditions, studies of validating reference genes have been driven by several algorithms and freely available software – geNorm (VANDESOMPELE *et al.* 2002), BestKeeper (PFAFFL *et al.* 2004), and NormFinder (ANDERSEN *et al.* 2004).

qPCR is generally characterise as an effective, sensitive, and economical methods, it has already widely applied to analyse gene expression in bio-

logical research (OVERBERGH 2003; LU *et al.* 2013; LIANG *et al.* 2014). However, there remain a number of problems have not yet been settled. One of the biggest challenges in qPCR analysis is normalisation of the variations arise from some mistakes in RNA extraction and purification, reverse transcription, efficiency of PCR amplification, etc. (BUSTIN *et al.* 2009). Several strategies have been processed to normalise these variations in qPCR analysis; these include normalisation of sample size, ensuring the quality and quantity of RNA, and removing DNA contamination (HUGGETT *et al.* 2005). Of such strategies, the most widely used is the selection of appropriate reference gene to normalise nonspecific variation or errors (LIANG *et al.* 2014). The expression of several conventional reference genes, including *18S RNA*,

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Supported by the National Key Research and Development Program of China, Project No. 2017YFD0201700, the Key Science and Technology Program of Henan (Agriculture), Project No. 182102110053, the Major Projects of Henan Institute of Science and Technology, China, Project No. 14QN024, the High-level Talent Introduction of Henan Institute of Science and Technology, China, Project No. 208010616003, the Scientific and Technological Innovation of Henan Institute of Science and Technology, China, Project No. 208010916005.

<https://doi.org/10.17221/20/2018-PPS>

*ACT*, and *GAPDH* has been demonstrated to change broadly under particular experimental conditions or in response to external stimuli (GLARE *et al.* 2002; MA *et al.* 2016). Clearly, for given a set of experimental biological samples, selecting suitable reference genes for use in the normalisation of qPCR data is quite urgent as several conventional reference genes are not always stable under all conditions.

Wild oat (*Avena fatua* L.) is a typical annual weeds of temperate agricultural regions in the world (HOLM *et al.* 1977). At the same time, it is also a malignant weed that harms wheat, oilseed rape, and other crops in China and has developed serious resistance to herbicides all over the world (CAVAN *et al.* 2001). In recent years, qPCR has been widely used to quantify gene expression levels in diverse studies of *A. fatua*, such as studies of herbicide resistance and ecological adaption (LI *et al.* 2009; CRUZHIPOLITO *et al.* 2011; KEITH *et al.* 2015). Some studies have shown that at least two or three reference genes should be used to achieve accurate normalisation (THELLIN *et al.* 1999; VANDESOMPELE *et al.* 2002). However, in the aforementioned studies, the researchers used only one reference gene (*18S*, *GAPDH*, or *ACT*) to normalise the variation in mRNA levels of genes of interest for all of the diverse experimental conditions. These less than ideal experimental practices likely resulted from a lack of empirical data about which reference genes in *A. fatua* are most appropriate for qPCR gene expression analysis. It is clear that several reference genes should ensure a more stable expression analysis. However, if only one suitable reference gene could be identified, this may be the case in a particular case. Much more important in such situations are the experimental conditions, the variations in the data and their interpretations.

The reference gene has seldom been verified systematically in weeds. A reference gene checking is quite urgent to ensure proper normalisation in *A. fatua*. These less than ideal experimental practices likely resulted from a lack of empirical data about which reference genes in *A. fatua* are most appropriate for qPCR gene expression analysis. We conducted the present study to ameliorate this situation and to enable the empirically informed selection of suitable reference genes for future studies with *A. fatua*. Eight commonly used normalisation genes (*18S*, *28S*, *ACT*, *GAPDH*, *EF1 $\alpha$* , *RPL7*,  *$\alpha$ -TUB*, and *TBP*) were selected for analysis of their performance under several different experimental conditions in *A. fatua*. After this analysis, two target genes, *HSP70*

and *AfatCYP71D7* were selected and used to validate the performance of the reference genes. Our results may offer some suggestion for the selection of suitable, reliable reference genes in modern molecular genetic analyses in *A. fatua*.

## MATERIAL AND METHODS

**Seeds of *A. fatua*.** Seeds of *A. fatua* used in this research were harvested in 2010 from wheat fields in Xinxiang of Henan Province, China, and had been stored for more than 7 years.

**Cultivation of seedlings.** The greenhouse potting methods of (LI *et al.* 2010) were adopted. The seeds of *A. fatua* were sown into pots with a surface area of 75 cm<sup>2</sup>. The soil surface with the unused herbicide was mixed with a proportion of grass biochar, sifted and cultured in the greenhouse. Rearing conditions were 20°C in the daytime and 15°C at night, 75 ± 5% relative humidity, and a 12 : 12 h light/dark photoperiod.

**Biotic factors.** To compare developmental stages, foliar parts from the 1-leaf, 2-leaf, and 3-leaf stage were collected in RNase-free tubes for each replication. The samples were collected in triplicate and then snap frozen in liquid nitrogen before being stored at –80°C for RNA extraction. Each experiment was completed using five plants (i.e., at least three biological replicates).

To compare different tissues, the roots, stems, and leaves from the 3-leaf stage were collected in RNase-free tubes for each replication. The samples were collected in triplicate and then snap frozen in liquid nitrogen before being stored at –80°C for RNA extraction. Each experiment was completed using five plants (i.e., at least three biological replicates).

**Abiotic factors.** A whole-plant assay modified was conducted according to RYAN (1970). Plants thinned and planted in the field (20 plants per pot) at the 3-leaf stage (20 cm plant height), were treated with fenoxaprop-*p*-ethyl by atomising with an auto spray device (Model ASP-1098, spray-head ST110-01, pressure 0.2 MPa). The herbicide was applied at a concentration of 10 g ai/ha (IC<sub>10</sub>) according to the results of a whole plant assay, the amount of spouting liquid was 450 l/ha. Water alone was used as a control. Each treatment was repeated three times. Foliar parts were collected for gene stability analyses at 24 h after treatment and stored at –80°C for RNA extraction.

**Reference gene selection and primer design.** Eight commonly used reference genes were selected, including 18S ribosomal RNA (rRNA) (*18S*), 28S rRNA (*28S*), actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor 1 alpha (*EF1 $\alpha$* ), ribosomal protein L7 (*RPL7*), Alpha-tubulin ( $\alpha$ -*TUB*), and TATA box binding protein-associated factor (*TBP*). Primer Premier 3.0 software was used to design the primers. Details on the primers used in this study are listed in Table 1.

**RNA extraction and cDNA synthesis.** To synthesise cDNA, RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Total RNA was (1  $\mu$ g) was reverse transcribed into First-strand complementary DNA using a PrimeScript RT reagent kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instructions and stored at  $-20^{\circ}\text{C}$  until use.

**Quantitative real-time PCR (qPCR).** ROX's Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) was used for qPCR and implementing on an

Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, USA). The reactions were performed in a 20  $\mu$ l volume of a mixture containing 1  $\mu$ l of cDNA template, 10  $\mu$ l of SYBR Green qPCR SuperMix-UDG, 0.3  $\mu$ l of each primer, and 8.7  $\mu$ l of nuclease-free water. The thermocycling program was as follows:  $50^{\circ}\text{C}$  for 2 min,  $95^{\circ}\text{C}$  for 2 min, and 40 cycles of  $95^{\circ}\text{C}$  for 15 s, and  $55^{\circ}\text{C}$  for 30 seconds. To acquire a high specificity amplification, a melting curve analysis between  $65^{\circ}\text{C}$  to  $95^{\circ}\text{C}$ , was constructed at the end of each PCR run. And it based on a 2-fold dilution series of cDNA (1 : 5, 1 : 10, 1 : 10, 1 : 10, 1 : 20, and 1 : 40). The corresponding qPCR efficiencies (E) were calculated refer to the formula  $E = 10^{-1/\text{slope}} - 1$  (PFAFFL 2001; TELLINGHUISEN 2014; SPIESS *et al.* 2015, 2016). Each sample was prepared as two biological replicates, and each reaction was analysed with three technical replications.

**Analysis of the stability of reference gene expression.** The expression stability of the eight selected reference genes was evaluated with the delta cycle

Table 1. Primers used in the study

Gene symbol	Gene name	T <sub>m</sub> (°C)	Sequence (5'–3')	Efficiency (%)	Product length (bp)	R <sup>2</sup>
<i>ACT</i>	actin	59.05 59.06	F: CATATGCGTCTTTCTGCCCC R: TGTGTGCGACAATGGAAGT	99.8	137	0.996
<i>18S</i>	18S ribosomal	59.00 58.83	F: TGCACCACCACCCATAGAAT R: CTGCGGCTTAATTTGACCCA	97.2	102	0.993
<i>28S</i>	28S ribosomal	58.27 59.02	F: ACCGGGCCTTAAAGCTACTT R: AAATGGAACCACTGCTGCTG	101.4	145	0.995
<i>GAPDH</i>	glyceraldehyde-3-phosphate	59.02 59.05	F: TCAAGCAAGGACTGGAGAGG R: AAGCTTGCCGTTCAACTCAG	108.3	111	0.999
<i>EF1<math>\alpha</math></i>	elongation factor 1 alpha	59.15 58.91	F: GTCTTGGTCGTCGCTTGC R: TTGTTGCAGGCGATGATCAG	90.5	125	0.993
<i>TBP</i>	TATA box binding protein	58.98 59.02	F: GATTTTAGCCTGCTCCCGTG R: ACGGCCCATATATCACCAGG	108.1	102	0.998
<i>RPL7</i>	ribosomal protein L7	59.09 59.00	F: AGGGTGGGTTCTATGTCAGC R: ATCTTCCTGGTCTTGGGGTG	95.6	91	0.997
$\alpha$ - <i>TUB</i>	Alpha-tubulin	59.12 59.10	F: GTGCTGGGAACTTTACTGCC R: TACTTGCTGCTCCAGTCTC	98.3	126	0.995
<i>HSP70</i>	heat shock protein 70	58.98 58.93	F: CTCGGCAAGTTTGAGCTCTC R: GGTGGCCTTATCTTTCGCAG	101.5	120	0.998
<i>AfatCY-P71D7</i>	cytochrome P450	58.90 59.13	F: TGTGCAAACGTCATTCCAGG R: GAATCTGGCTCGGTGCGACTA	100.6	92	0.998

F – forward primer; R – reverse primer; T<sub>m</sub> – melting temperature; R<sup>2</sup> – coefficient of determination

<https://doi.org/10.17221/20/2018-PPS>

threshold (Ct) method ( $\Delta$ Ct method) and three commonly used software tools: geNorm v3.5 (VANDESOMPELE *et al.* 2002), Normfinder v0.953 (ANDERSEN *et al.* 2004), and BestKeeper (PFAFFL *et al.* 2004). The geNorm software initially calculates the value of gene expression stability (M) and generates a stability ranking; genes with the lowest M value have the most stable expression. Accurate normalisation of two sequential factors is essential, which is generated by calculates pairwise variation  $V_{n/n+1}$  through geNorm. A  $V_{n/n+1}$  ratio below 0.15 suggest that the use of an additional reference gene would not significantly improve normalisation. NormFinder software is a model-based approach to identifying suitable reference genes for use in normalisation (ANDERSEN *et al.* 2004). The candidate gene with the lowest value is considered to be the most stable. The MS Excel-based software BestKeeper and  $\Delta$ Ct method were also used to select optimal reference genes. A user-friendly web based comprehensive tool, RefFinder online (<http://150.216.56.64/referencegene.php>) to evaluate and select reference genes. RefFinder combines the aforementioned major computational programs ( $\Delta$ Ct method, geNorm, Normfinder, and BestKeeper) to compare and rank the tested candidate reference genes. And it also assigns an appropriate weight to each gene and calculates the geometric mean of the weights for the final ranking.

**Validation of reference gene selection.** To evaluate the validity of the optimised selection of reference genes, expression levels of the heat shock protein 70 gene (*HSP70*) and *AfatCYP71D7* were analysed under different experimental conditions (different tissues, developmental stages and herbicide treatments). For each experimental condition, the expression profiles of the gene *HSP70* and *AfatCYP71D7* were normalised using only one reference gene (the most stable reference gene [NF1] and the least stable reference gene [NF8]) and several stable reference genes (NF(1-n)) recommended by RefFinder. The relative expression levels of *HSP70* and *AfatCYP71D7* in different samples were calculated following the  $2^{-\Delta\Delta C_t}$  method (PFAFFL 2001).

**Statistical analysis.** Data statistics and bioassay analysis were performed on MS Excel (2010) and Polo (Probit and Logit Analysis) (LeOra Software Company, Petaluma, USA). The target gene expression normalised by the least stable reference gene, and the recommended combination of reference genes were calculated by one-way ANOVA using the software InStat v3.0 (GraphPad Software, San Diego, USA) with a significance level set at  $P < 0.05$ .

## RESULTS

### PCR amplification efficiencies and expression levels of candidate reference genes

Traditional PCR was used to evaluate the primer specificity of the eight reference genes and the one target gene of interest used. Melting curve analysis showed that there were single peaks for each primer pair, which further demonstrated that each primer pair amplified a unique product. A standard curve was generated for each gene using five-fold serial dilutions of cDNA. The amplification efficiencies of all the primer pairs were between 90.5% and 108.1%, and the coefficient of determination ( $R^2$ ) ranged from 0.993 to 0.999 (Table 1).

The Ct values have been created to compare the transcript abundance of the selected genes in different samples. The mean Ct values of the eight reference genes varied significantly. The means of the Ct values ranged from 12.35 to 25.52, with the lowest and highest Ct values obtained from *18S* (12.35) and *RPL7* (25.52). *RPL7* ( $25.52 \pm 0.43$ ) had the highest mean expression levels, followed by *EF1 $\alpha$*  ( $23.76 \pm 0.25$ ),  *$\alpha$ -TUB* ( $23.54 \pm 0.35$ ), *GAPDH* ( $22.76 \pm 0.31$ ), *TBP* ( $21.97 \pm 0.27$ ), *ACT* ( $21.75 \pm 0.30$ ), *28S* ( $21.11 \pm 0.30$ ), and *18S* ( $12.35 \pm 0.26$ ) (Figure 1).

### Expression stability of the candidate reference genes

**Developmental stages.** The overall expression stability rankings produced by the two methods ( $\Delta$ Ct method and NormFinder) were almost identical, the top two stable reference genes were *28S* and *EF1 $\alpha$* . Interestingly, *EF1 $\alpha$*  was identified by BestKeeper as

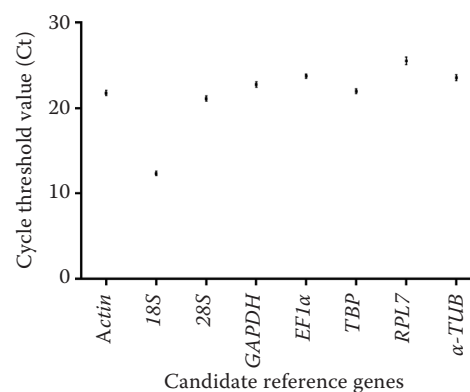


Figure 1. Expression levels of candidate reference genes of *A. fatua*

Table 2. Expression stability of the candidate reference genes under different experimental conditions

Conditions	$\Delta Ct$		BestKeeper		Normfinder		geNorm	
	stability	rank	stability	rank	stability	rank	stability	rank
Developmental sages	0.322	3	0.208	3	0.186	3	0.668	3
	0.272	1	0.182	2	0.066	1	0.663	2
	0.303	2	0.278	6	0.133	2	0.673	4
	0.365	7	0.357	7	0.264	7	0.943	6
	0.630	8	0.489	8	0.605	8	1.290	8
	0.351	6	0.263	5	0.251	6	0.640	1
	0.332	4	0.226	4	0.242	5	0.743	5
	0.339	5	0.142	1	0.199	4	1.130	7
Different tissues	0.88	5	1.198	2	0.521	6	0.836	2
	0.785	1	1.407	4	0.101	1	0.861	3
	0.872	4	1.083	1	0.462	5	0.796	1
	1.225	6	1.248	3	1.118	3	1.004	6
	1.766	8	1.456	6	1.613	8	0.892	4
	0.833	2	1.432	5	0.374	4	0.959	5
	0.840	3	1.508	7	0.111	2	1.049	7
	1.725	7	2.031	8	1.570	7	1.270	8
Herbicide treatments	0.293	2	1.065	4	0.063	2	0.308	3
	0.402	6	1.290	8	0.349	7	0.148	1
	0.439	7	0.798	2	0.294	6	0.309	4
	0.306	3	1.038	3	0.109	3	0.819	6
	0.738	8	0.523	1	0.715	8	1.179	8
	0.286	1	1.078	5	0.056	1	0.244	2
	0.355	5	1.163	6	0.224	5	0.379	5
	0.313	4	1.173	7	0.191	4	0.989	7
Pooled samples	0.667	3	0.836	2	0.308	3	0.323	3
	0.662	2	0.861	3	0.148	1	0.368	4
	0.675	4	0.796	1	0.309	4	0.306	1
	0.940	6	1.004	6	0.819	6	0.516	6
	1.295	8	0.892	4	1.179	8	0.840	8
	0.640	1	0.959	5	0.244	2	0.309	2
	0.743	5	1.049	7	0.379	5	0.435	5
	1.133	7	1.270	8	0.989	7	0.690	7

the less unstably expressed reference gene, *RPL7* and *28S* was identified by BestKeeper as the most stably expressed reference genes. The geNorm ranked *GAPDH* and *28S* as the top two stable reference genes (Table 2). According to the RefFinder method, the stability rankings from the most stable to the least stable across different developmental stages were as follows: *28S*, *EF1 $\alpha$* , *RPL7*, *TBP*,  *$\alpha$ -TUB*, *GAPDH*, *ACT*, and *18S* (Figure 2A). For geNorm analysis, all of the  $V_{n/n+1}$  values were below the threshold of  $P < 0.15$  (Figure 3), indicating that the two most stable genes

are required for normalisation. Therefore, for the developmental stage experiments, *28S* and *EF1 $\alpha$*  were appropriate to normalisation.

**Different tissues.** The stability rankings produced by BestKeeper, and geNorm were similar, that *EF1 $\alpha$*  and *TBP* were confirmed as the two most stably expressed reference genes. However,  $\Delta Ct$  method and NormFinder analysis indicated *EF1 $\alpha$*  and *TBP* were less unstably expressed reference genes.  $\Delta Ct$  method identified *28S* and *GAPDH*, and NormFinder identified *28S* and  *$\alpha$ -TUB* as the most stably expressed



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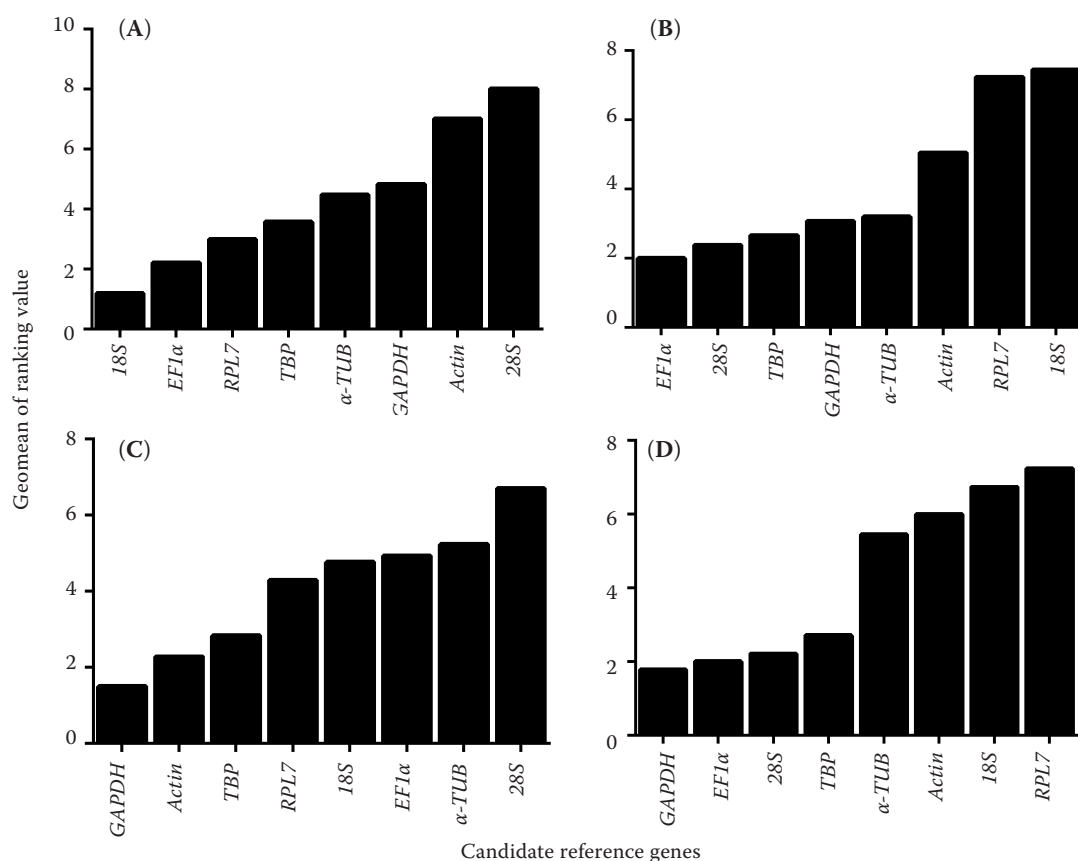


Figure 2. Expression stability of the candidate reference genes under different experimental conditions: (A) development stage, (B) tissues, (C) herbicide treatments, and (D) all samples

reference genes (Table 2). According to the RefFinder method, the stability rankings from the most stable to the least stable across different developmental stages were as follows: *EF1 $\alpha$* , *28S*, *TBP*, *GAPDH*,  *$\alpha$ -TUB*, *ACT*, *RPL7*, and *18S* (Figure 2B). For geNorm analysis, the  $V_{2/3}$  was below the threshold of  $P < 0.15$  (Figure 3). Thus, two reference genes were enough to normalise the gene expression levels in qPCR analyses. Therefore, *EF1 $\alpha$*  and *28S* were the most suitable for normalising qPCR data in the different tissues.

**Herbicide treatments.**  $\Delta$ Ct method and NormFinder identified *GAPDH* and *TBP* as the most stably expressed reference genes, *18S* was identified as the least stably expressed reference gene. However, BestKeeper identified *18S* and *EF1 $\alpha$* , and geNorm identified *28S* and *GAPDH* as the most stably expressed reference genes (Table 2). According to the RefFinder method, the stability rankings from the most stable to the least stable across herbicide treatments were as follows: *GAPDH*, *ACT*, *TBP*, *RPL7*, *18S*, *EF1 $\alpha$* ,  *$\alpha$ -TUB*, and *28S* (Figure 2C). For geNorm analysis, all of the  $V_{n/n+1}$  values were below the 0.15 cut-off value following geNorm analysis (Figure 3). *GAPDH*

and *ACT* were suggested for normalising the qPCR data in the herbicide treatments.

**Pooled data of various conditions.** The stability rankings produced by Ct methods and NormFinder were similar, that *28S* and *GAPDH* were confirmed as the two most stably expressed reference genes. However, BestKeeper identified *EF1 $\alpha$*  and *TBP*, and geNorm identified *EF1 $\alpha$*  and *GAPDH* as the most stably expressed reference genes (Table 2). According to the RefFinder method, the stability rankings from the most stable to the least stable across pooled data of various conditions were as follows: *GAPDH*, *EF1 $\alpha$* , *28S*, *TBP*,  *$\alpha$ -TUB*, *ACT*, *18S*, and *RPL7* (Figure 2D). For geNorm analysis, all of the  $V_{n/n+1}$  values were below the 0.15 cut-off value following geNorm analysis (Figure 3). *GAPDH* and *EF1 $\alpha$*  was suggested for normalising the qPCR data in pooled data of various conditions.

### Validation of reference gene selection

To distinguish the performance of selected reference genes, the expression level of *HSP70* and *Afat-*

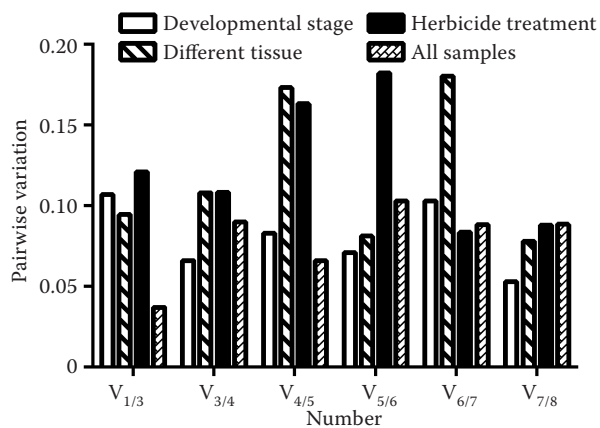


Figure 3. Optimal number of reference genes for normalisation in *A. fatua*

*CYP71D7* was analysed in the same experimental conditions used to compare the expression stability of the reference genes. Similar expression levels were obtained in the developmental stage experiments when normalised using the most stable reference gene (*28S*) and the combination of the two most stable reference genes (*28S* and *EF1 $\alpha$* ), and *HSP70* transcript levels were higher in 3-leaf stage compared with both 1-leaf stage and 2-leaf stage. In addition, when normalised with the least stable reference gene (*18S*), the *HSP70* transcript levels were also higher in 3-leaf stage compared with both 1-leaf stage and 2-leaf stage (Figure 4A). For the experiments with different tissues, the *HSP70* transcript level was higher in the stems than in the other two tissues, no matter whether it was normalised by the most stable reference gene (*EF1 $\alpha$* ), the combination of the two most stable reference genes (*EF1 $\alpha$*  and *28S*), or the least stable reference gene (*18S*). However, the *HSP70* transcript level was significantly higher when normalised by the least stable reference gene (*18S*) than by the most suitable reference gene (*EF1 $\alpha$* ) and the combination of recommended reference genes (*EF1 $\alpha$*  and *28S*) in the stems. The expression levels of *HSP70* normalised using the most stable reference gene were not different from those using the combination of recommended reference genes and the least stable reference gene in the tissues of leaves (Figure 4B). The expression profiles of *HSP70* were not significantly different in the herbicide treatments, no matter whether the most stable reference gene (*GAPDH*), the combination of the two most stable reference genes (*GAPDH* and *ACT*) or the least stable reference gene (*28S*) was used for the normalisation. The *HSP70* expression levels were higher in the treatment groups than the control groups and

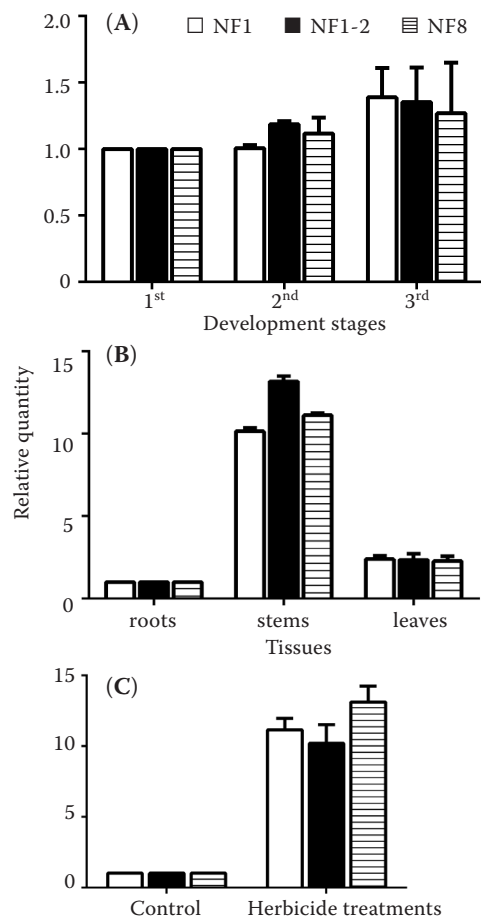


Figure 4. Relative expression levels of a target gene of interest (*HSP70*) were calculated using different sets of reference genes

were significantly higher when normalised by the least stable reference gene (*28S*) than by the most suitable reference gene (*GAPDH*) and the combination of recommended reference genes (*GAPDH* and *ACT*) (Figure 4C).

Another gene, *AfatCYP71D7*, which transcript level was lowest in 1-leaf stage while it was the highest in 3-leaf stage among all developmental stages, and no evident difference was observed among all developmental stages. Furthermore, the expression level of *AfatCYP71D7* normalised by the most stable reference gene (*28S*) or the combination of the two best reference genes (*28S* and *EF1 $\alpha$* ) was not significantly different from the expression level calculated using the least suitable reference gene (*18S*) in each developmental stage ( $P < 0.05$ ) (Figure 5A). Across different tissues, which transcript level was lowest in the roots while it was the highest in the stems among all the tissues, and no evident difference of *AfatCYP71D7* transcript levels was observed in the

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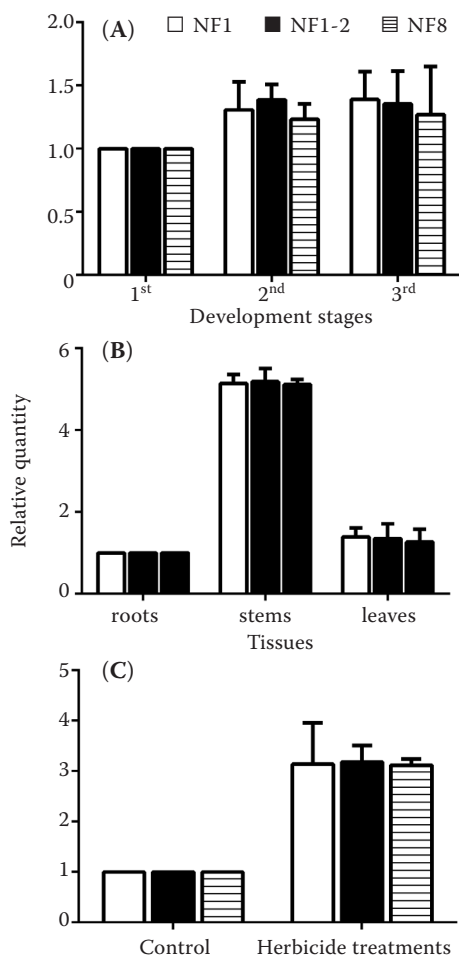


Figure 5. Relative expression levels of a target gene of interest (*AfatCYP71D7*) were calculated using different sets of reference genes

leaves no matter whether it was normalised by the most stable reference gene (*EF1 $\alpha$* ), the combination of the two most stable reference genes (*EF1 $\alpha$*  and *28S*), or the least stable reference gene (*18S*). However, the expression level of *HSP70* normalised by the most stable reference gene (*EF1 $\alpha$* ), the combination of the two most stable reference genes *EF1 $\alpha$*  and *28S* was markedly different from the expression level calculated using the least stable reference gene (*18S*) in the stems (Figure 5B). Across herbicide treatments, *AfatCYP71D7* transcript levels increased significantly in herbicide treatments compared with controls no matter whether it was normalised by the most stable reference gene (*GAPDH*), the combination of the two most stable reference genes (*GAPDH* and *ACT*), or the least stable reference gene (*28S*). Furthermore, the expression level of *AfatCYP71D7* normalised by the most stable reference gene or the combination of the two best reference genes was not markedly

different from the expression level calculated using the least suitable reference gene in treatments ( $P < 0.05$ ) (Figure 5C).

## DISCUSSION

To our knowledge, this is first systematic study to validate a set of candidate reference genes for qPCR in *A. fatua*. Our results indicated that *GAPDH* and *EF1 $\alpha$*  were the best reference genes according to the average expression stability (M) or stability values acquired by  $\Delta C_t$  method, geNorm, BestKeeper, Normfinder and RefFinder. There were some differences in developmental stages, tissues and herbicide treatments when the outcomes of the five methods were compared. Considering the developmental stages, the most stable genes were *28S* and *EF1 $\alpha$*  ( $\Delta C_t$  method, NormFinder and RefFinder), *RPL7* and *28S* (Best-keeper), *GAPDH* and *28S* (geNormal), respectively. Among different tissues, the most stable genes were *EF1 $\alpha$*  and *TBP* (BestKeeper and geNorm), *28S* and *GAPDH* ( $\Delta C_t$  method), *28S* and  *$\alpha$ -TUB* (NormFinder), and *EF1 $\alpha$*  and *28S* (RefFinder), respectively. For herbicide treatments, the most stable genes were *GAPDH* and *TBP* ( $\Delta C_t$  methods and NormFinder), *18S* and *EF1 $\alpha$*  (BestKeeper), *28S* and *GAPDH* (geNorm), and *GAPDH* and *ACT* (RefFinder), respectively. Based on the rankings from RefFinder, which integrates outcomes of the four major statistic algorithms ( $\Delta C_t$  methods, geNorm, Normfinder, and Bestkeeper), and it also assigns an appropriate weight to an individual gene and calculates the geometric mean of their weight, *GAPDH* and *EF1 $\alpha$*  had a good performance under specific conditions. Of these reference genes tested (*GAPDH*, *EF1 $\alpha$* , *18S*, *28S*, and *ACT*) in *A. fatua* varied greatly, *GAPDH* and *EF1 $\alpha$*  were recommended as the most suitable reference genes while *18S* was ranked as the less suitable reference genes under the majority of the experimental conditions in our results, which was consistent with the other studies examining reference gene expression (PETIT *et al.* 2012; DUHOUX & DÉLYE 2013). However, some reports indicated *18S* was recommended to validate gene expression data in *Solanum melongena* L. or *Oryza sativa* L. (KIM *et al.* 2003; GANTASALA *et al.* 2013). *GAPDH* was one of the most stably expressed genes in our results consistent with the previous results in *A. fatua* (WRZESIŃSKA *et al.* 2016) as well as other weed species, such as *Alopecurus myosuroides* Huds and *Lolium* sp. (DUHOUX & DÉLYE 2013). However, *GAPDH* was not stable in



*Petunia x hybrida* during leaf and flower development (MALLONA *et al.* 2010). *EF1 $\alpha$*  was also recommended as one of the most stable reference gene to validate gene expression data in this study, the similar results were obtained in other researches (HORNÁKOVÁ *et al.* 2010; ZHANG *et al.* 2015).

The suitability rankings of the reference genes were different with the different programs as the stability of the expression of eight candidate reference genes was evaluated via five commonly used programs (RefFinder, geNorm, NormFinder, BestKeeper, and  $\Delta$ Ct method) for data generated in different experimental conditions. Thus, *28S*, *TBP*, *ACT* and so on in specific conditions were also recommended as suitable for reference genes. This was somewhat consistent with the previous results *18S* or *28S* is an ideal reference gene for normalisation of qPCR data (BAGNALL & KOTZE 2010). *TBP* is as one of the most stably expressed genes in previous study of *A. fatua* (WRZESIŃSKA *et al.* 2016) as well as the research in *Solanum lycopersicum* and *Lolium multiflorum* L. (EXPÓSITO-RODRÍGUEZ *et al.* 2008; WANG *et al.* 2015). *ACT* as traditional reference genes is not always stable in different experimental conditions, which was somewhat in accordance with several studies demonstrating *ACT* as an unsuitable internal control for RT-PCR in other cell types or species (LORD *et al.* 2010). Recent studies have shown that *ACT* expression can change in response to a variety of conditions (RUAN & LAI 2007; ZARIVI *et al.* 2015; WRZESIŃSKA *et al.* 2016). Thus, these traditionally used reference genes are not persistently and stably expressed in many species or different experimental treatments (CHANDNA 2012; CHENG *et al.* 2013; MA *et al.* 2016), which emphasised the need to evaluate reference genes in *A. fatua*.

To validate whether these selected reference genes are reliable in the conditions tested in this study, the expression levels of *HSP70*, an important stress-inducible heat shock protein gene (BETTENCOURT *et al.* 2007) and *AfatCYP71D7*, a P450 gene that could be induced significantly by herbicides in our previous results, were analysed in different developmental stages, tissues, and herbicide treatments, demonstrating that the use of unsuitable reference gene for normalisation might lead to deviated results. Therefore, it has proved that choosing appropriate reference genes for normalisation is a key precondition for the accurate estimation of target gene expression though only two target genes were chosen to validate these selected reference genes.

In summary, eight genes were tested via five popularly applied programs and confirmed that *GAPDH* and *EF1 $\alpha$*  were the most suitable reference genes for explore gene expression profiles of different developmental stages, tissues, and herbicide treatments. This study not only provides useful reference to Northern blot and reverse transcription PCR techniques that require a reference gene for normalisation, but also identifies several potential reference genes to accurately evaluate target gene expression profiles in *A. fatua*.

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Received: February 3, 2018

Accepted: July 23, 2018

Published online: August 10, 2018