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

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**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 biological 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,
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 (Thelin 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, EFla, RPL7, a-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 cm². 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 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 (IC₅₀) 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 (EF1α), ribosomal protein L7 (RPL7), Alpha-tubulin (α-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 synthesize cDNA, RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. Total RNA was (1 µg) 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°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 µl volume of a mixture containing 1 µl of cDNA template, 10 µl of SYBR Green qPCR SuperMix-UDG, 0.3 µl of each primer, and 8.7 µl of nuclelease-free water. The thermocycling program was 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 seconds. To acquire a high specificity amplification, a melting curve analysis between 65°C to 95°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 : 20, and 1 : 40). The corresponding qPCR efficiencies (E) were calculated refer to the formula \( E = 10^{\frac{1}{\text{slope}} - 1} \) (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

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Tm (°C)</th>
<th>Sequence (5’–3’)</th>
<th>Efficiency (%)</th>
<th>Product length (bp)</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT</td>
<td>actin</td>
<td>59.05</td>
<td>F: CATATGCCTCTTTCTGGCCCCC</td>
<td>99.8</td>
<td>137</td>
<td>0.996</td>
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<tr>
<td></td>
<td></td>
<td>59.06</td>
<td>R: TGTTGGCGGAAATGGAACTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S</td>
<td>18S ribosomal</td>
<td>59.00</td>
<td>F: TGGACCACCACCACCATAGAAT</td>
<td>97.2</td>
<td>102</td>
<td>0.993</td>
</tr>
<tr>
<td></td>
<td></td>
<td>58.83</td>
<td>R: CTGCGGCTTAATTGTGACCCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28S</td>
<td>28S ribosomal</td>
<td>58.27</td>
<td>F: ACCGGGCTTTAAAGCTACTT</td>
<td>101.4</td>
<td>145</td>
<td>0.998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59.02</td>
<td>R: AATGGAACCACCTGCTGCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate</td>
<td>59.02</td>
<td>F: TCAGCAAGGACTGGAGAGG</td>
<td>108.3</td>
<td>111</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59.05</td>
<td>R: AAGCTTGGCGGTCGACTCAG</td>
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<tr>
<td>EF1α</td>
<td>elongation factor 1 alpha</td>
<td>59.15</td>
<td>F: GTCTTGTCGTCGCTTG</td>
<td>90.5</td>
<td>125</td>
<td>0.993</td>
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<td></td>
<td></td>
<td>58.91</td>
<td>R: TGTTGCAAGGCGATGATCAG</td>
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<tr>
<td>TBP</td>
<td>TATA box binding protein</td>
<td>58.98</td>
<td>F: GATTTTAGCCTGCTCCCGTG</td>
<td>108.1</td>
<td>102</td>
<td>0.998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59.02</td>
<td>R: ACGGCCCATATATCACCCAGG</td>
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<td>RPL7</td>
<td>ribosomal protein L7</td>
<td>59.09</td>
<td>F: AGGGTGCGTCTCTTG</td>
<td>95.6</td>
<td>91</td>
<td>0.997</td>
</tr>
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<td></td>
<td></td>
<td>59.00</td>
<td>R: ATCTTCTTCTGCTTGGGGTG</td>
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<td></td>
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<tr>
<td>α-TUB</td>
<td>Alpha-tubulin</td>
<td>59.12</td>
<td>F: GTGCTGGGAACATTACTGCC</td>
<td>98.3</td>
<td>126</td>
<td>0.998</td>
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<td></td>
<td></td>
<td>59.10</td>
<td>R: TACCTTGGCTGCTCCAGTCTC</td>
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<tr>
<td>HSP70</td>
<td>heat shock protein 70</td>
<td>58.98</td>
<td>F: CTCGGCAGTGGCTGCTCT</td>
<td>101.5</td>
<td>120</td>
<td>0.998</td>
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<td></td>
<td></td>
<td>58.93</td>
<td>R: GGTTGGGCTTATTTTGGCAG</td>
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<td></td>
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<tr>
<td>AfatCY-P71D7</td>
<td>cytochrome P450</td>
<td>58.90</td>
<td>F: TGTTGCAAAGCGTATTCCAGG</td>
<td>100.6</td>
<td>92</td>
<td>0.998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59.13</td>
<td>R: GAATCTGGCGGCTGCTGACTA</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

F – forward primer; R – reverse primer; Tm – melting temperature; \( R^2 \) – coefficient of determination
threshold (Ct) method (ΔCt 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 Vn/n+1 through geNorm. A Vn/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 ΔCt 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 (ΔCt 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 A. fatua CYP71D7 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 A. fatua CYP71D7 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 A. fatua CYP71D7 in different samples were calculated following the $2^{-\Delta\Delta\text{Ct}}$ 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 ± 0.43) had the highest mean expression levels, followed by EF1α (23.76 ± 0.25), a-TUB (23.54 ± 0.35), GAPDH (22.76 ± 0.31), TBP (21.97 ± 0.27), ACT (21.75 ± 0.30), 28S (21.11 ± 0.30), and 18S (12.35 ± 0.26) (Figure 1).

Expression stability of the candidate reference genes

Developmental stages. The overall expression stability rankings produced by the two methods (ΔCt method and NormFinder) were almost identical, the top two stable reference genes were 28S and EF1α. Interestingly, EF1α was identified by BestKeeper as
the less unstably expressed reference gene, \textit{RPL7} and \textit{28S} was identified by BestKeeper as the most stably expressed reference genes. The \textit{geNorm} ranked \textit{GAPDH} and \textit{28S} as the top two stable reference genes (Table 2). According to the \textit{RefFinder} method, the stability rankings from the most stable to the least stable across different developmental stages were as follows: \textit{28S}, \textit{EF1\alpha}, \textit{RPL7}, \textit{TBP}, \textit{\alpha-TUB}, \textit{GAPDH}, \textit{ACT}, and \textit{18S} (Figure 2A). For \textit{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, \textit{28S} and \textit{EF1\alpha} were appropriate to normalisation.

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

\begin{table}[h]
\centering
\caption{Expression stability of the candidate reference genes under different experimental conditions}
\label{tab:2}
\begin{tabular}{llllllll}
\hline
\textbf{Conditions} & \textbf{\(\Delta \text{Ct}\)} & \textbf{BestKeeper} & \textbf{Normfinder} & \textbf{\textit{geNorm}} \\
 & & \textbf{stability} & \textbf{rank} & \textbf{stability} & \textbf{rank} & \textbf{stability} & \textbf{rank} \\
\hline
\textbf{Developmental sages} & & & & & & & \\
\textit{RPL7} & 0.322 & 3 & 0.208 & 3 & 0.186 & 3 & 0.668 & 3 \\
\textit{28S} & 0.272 & 1 & 0.182 & 2 & 0.066 & 1 & 0.663 & 2 \\
\textit{GAPDH} & 0.303 & 2 & 0.278 & 6 & 0.133 & 2 & 0.673 & 4 \\
\textit{28S} & 0.365 & 7 & 0.357 & 7 & 0.264 & 7 & 0.943 & 6 \\
\textit{GAPDH} & 0.630 & 8 & 0.489 & 8 & 0.605 & 8 & 1.290 & 8 \\
\textit{EF1\alpha} & 0.351 & 6 & 0.263 & 5 & 0.251 & 6 & 0.640 & 1 \\
\textit{28S} & 0.332 & 4 & 0.226 & 4 & 0.242 & 5 & 0.743 & 5 \\
\textit{18S} & 0.339 & 5 & 0.142 & 1 & 0.199 & 4 & 1.130 & 7 \\
\hline
\textbf{Different tissues} & & & & & & & \\
\textit{GAPDH} & 0.88 & 5 & 1.198 & 2 & 0.521 & 6 & 0.836 & 2 \\
\textit{EF1\alpha} & 0.785 & 1 & 1.407 & 4 & 0.101 & 1 & 0.861 & 3 \\
\textit{28S} & 0.872 & 4 & 1.083 & 1 & 0.462 & 5 & 0.796 & 1 \\
\textit{GAPDH} & 1.225 & 6 & 1.248 & 3 & 1.118 & 3 & 1.004 & 6 \\
\textit{EF1\alpha} & 1.766 & 8 & 1.456 & 6 & 1.613 & 8 & 0.892 & 4 \\
\textit{TBP} & 0.833 & 2 & 1.432 & 5 & 0.374 & 4 & 0.959 & 5 \\
\textit{28S} & 0.840 & 3 & 1.508 & 7 & 0.111 & 2 & 1.049 & 7 \\
\textit{EF1\alpha} & 1.725 & 7 & 2.031 & 8 & 1.570 & 7 & 1.270 & 8 \\
\hline
\textbf{Herbicide treatments} & & & & & & & \\
\textit{GAPDH} & 0.293 & 2 & 1.065 & 4 & 0.063 & 2 & 0.308 & 3 \\
\textit{EF1\alpha} & 0.402 & 6 & 1.290 & 8 & 0.349 & 7 & 0.148 & 1 \\
\textit{28S} & 0.439 & 7 & 0.798 & 2 & 0.294 & 6 & 0.309 & 4 \\
\textit{GAPDH} & 0.306 & 3 & 1.038 & 3 & 0.109 & 3 & 0.819 & 6 \\
\textit{EF1\alpha} & 0.738 & 8 & 0.523 & 1 & 0.715 & 8 & 1.179 & 8 \\
\textit{TBP} & 0.286 & 1 & 1.078 & 5 & 0.056 & 1 & 0.244 & 2 \\
\textit{28S} & 0.355 & 5 & 1.163 & 6 & 0.224 & 5 & 0.379 & 5 \\
\textit{EF1\alpha} & 0.313 & 4 & 1.173 & 7 & 0.191 & 4 & 0.989 & 7 \\
\hline
\textbf{Pooled samples} & & & & & & & \\
\textit{GAPDH} & 0.667 & 3 & 0.836 & 2 & 0.308 & 3 & 0.323 & 3 \\
\textit{EF1\alpha} & 0.662 & 2 & 0.861 & 3 & 0.148 & 1 & 0.368 & 4 \\
\textit{28S} & 0.675 & 4 & 0.796 & 1 & 0.309 & 4 & 0.306 & 1 \\
\textit{GAPDH} & 0.940 & 6 & 1.004 & 6 & 0.819 & 6 & 0.516 & 6 \\
\textit{EF1\alpha} & 1.295 & 8 & 0.892 & 4 & 1.179 & 8 & 0.840 & 8 \\
\textit{TBP} & 0.640 & 1 & 0.959 & 5 & 0.244 & 2 & 0.309 & 2 \\
\textit{28S} & 0.743 & 5 & 1.049 & 7 & 0.379 & 5 & 0.435 & 5 \\
\textit{\alpha-TUB} & 1.133 & 7 & 1.270 & 8 & 0.989 & 7 & 0.690 & 7 \\
\hline
\end{tabular}
\end{table}
According to the RefFinder method, the stability rankings from the most stable to the least stable across different developmental stages were as follows: EF1α, 28S, TBP, GAPDH, α-TUB, ACT, RPL7, and 18S (Figure 2B). For geNorm analysis, the V_{2/n} 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, EF1α and 28S were the most suitable for normalising qPCR data in the different tissues.

**Herbicide treatments.** ΔCt 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 EF1α, 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, EF1α, α-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 EF1α and TBP, and geNorm identified EF1α 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, EF1α, 28S, TBP, α-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 EF1α 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 Atf-
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 EF1α), 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 (EF1α), the combination of the two most stable reference genes (EF1α 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 (EF1α) and the combination of recommended reference genes (EF1α 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 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 EF1α) 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

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

Figure 4. Relative expression levels of a target gene of interest (HSP70) were calculated using different sets of reference genes
leaves no matter whether it was normalised by the most stable reference gene (EF1α), the combination of the two most stable reference genes (EF1α and 28S), or the least stable reference gene (18S). However, the expression level of HSP70 normalised by the most stable reference gene (EF1α), the combination of the two most stable reference genes EF1α 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 EF1α were the best reference genes according to the average expression stability (M) or stability values acquired by ΔCt 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 EF1α (ΔCt method, NormFinder and RefFinder), RPL7 and 28S (Bestkeeper), GAPDH and 28S (geNormal), respectively. Among different tissues, the most stable genes were EF1α and TBP (BestKeeper and geNorm), 28S and GAPDH (ΔCt method, NormFinder, 28S and a-TUB (NormFinder), and EF1α and 28S (RefFinder), respectively. For herbicide treatments, the most stable genes were GAPDH and TBP (ΔCt methods and NormFinder), 18S and EF1α (BestKeeper), 28S and GAPDH (geNormal), and GAPDH and ACT (RefFinder), respectively. Based on the rankings from RefFinder, which integrates outcomes of the four major statistic algorithms (ΔCt methods, geNorm, Normfinder, and Bestkeeper), it also assigns an appropriate weight to an individual gene and calculates the geometric mean of their weight, GAPDH and EF1α had a good performance under specific conditions. Of these reference genes tested (GAPDH, EF1α, 18S, 28S, and ACT) in *A. fatua* varied greatly, GAPDH and EF1α 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

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

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Petunia x hybrida during leaf and flower development (Mallona et al. 2010). EF1α 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 ΔCt 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 AjatCYP71D7, 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 EF1α 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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