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Identification of Reference Genes for Studying Herbicide Resistance Mechanisms in Japanese Foxtail (*Alopecurus japonicus*)

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Herbicide-resistant weeds pose a considerable threat to agriculture, but their resistance mechanisms are poorly understood. Differential gene expression analysis of a weed subjected to herbicide treatment is a key step toward more mechanistic studies. Such an analysis, often involving quantitative real-time PCR (qPCR), requires suitable reference genes as internal controls. In this study, we identified optimal reference genes in the noxious weed, Japanese foxtail. This weed has evolved resistance to acetyl-coenzyme A carboxylase (ACCase) inhibitors. We analyzed the stability of eight commonly used candidate reference genes (*glyceraldehyde-3-phosphate dehydrogenase [GAPDH]; ubiquitin [UBQ]; capsine phosphatase [CAP]; beta-tubulin [TUB]; eukaryotic initiation factor 4a [EIF4A]; elongation factor-1 alpha [EF1]; 18S ribosomal RNA [18S]; 25S ribosomal RNA [25S]*) from root, stem, and leaf tissue of plants that were either resistant or sensitive to ACCase inhibitors, with or without herbicide stress, using qPCR. The results were further ranked and analyzed using geNorm, NormFinder, and BestKeeper software. These analyses identified *EF1* and *UBQ* in roots, *EF1*, *TUB*, *CAP*, and *18S* in stems, and *EF1*, *GAPDH*, and *18S* in leaves as suitable references for qPCR normalization. We have identified a set of reference genes that can be used to study herbicide resistance mechanisms in Japanese foxtail.
Nomenclature: Japanese foxtail, *Alopecurus japonicus* Steud.
Key words: ACCase, BestKeeper, fenoxaprop-P-ethyl, gene expression, geNorm, NormFinder, qPCR.

Japanese foxtail, a monocot weed from the Poaceae family, is one of the most economically important weeds in Asia. This weed can cause great harm to cereal and oilseed rape (*Brassica napus* L.) production. In weed management systems, Japanese foxtail control mainly depends on chemical herbicides. However, this weed has evolved resistance to many classes of herbicides, such as aryloxyphenoxypropionates, phenylpyrazolins, triazolopyrimidines, and sulfonylureas (Mohamed et al. 2012; Tang et al. 2012; Xu et al. 2013, 2014; Yang et al. 2007). Target-site resistance (TSR) and/or non–target site resistance (NTSR) mechanisms have evolved in a great number of weed species (Délye 2005; Powles and Yu 2010). TSR mechanisms include increased expression of target proteins or structural changes to the herbicide-binding sites (Délye et al. 2013). NTSR mechanisms mainly reduce the number of active herbicide molecules reaching their target (Powles and Yu 2010; Yuan et al. 2007). In Japanese foxtail, acetyl-coenzyme A carboxylase (ACCase) structural changes have been implicated in the resistance mechanism (Mohamed et al. 2012; Tang et al. 2012; Xu et al. 2013, 2014). Nevertheless, whether differential gene expression of *ACCase* could also play a role in conferring resistance in Japanese foxtail is still unknown.

Herbicide treatment is a "special" abiotic stress to weed species. As the regulation of gene expression mediates the plant's response to environmental stress, understanding the molecular regulation mechanisms in these physiological and ecological adaptations in Japanese foxtail may help to decipher the resistance mechanisms and provide insights into regulatory networks in weeds (Délye et al. 2013; Duhoux and Délye 2013). qPCR has become the most prevalent method for determining changes in gene expression due to its accuracy, specificity, efficiency, and reproducibility (Nolan et al. 2006; Schmittgen and Livak 2008). The most accepted approach is relative quantification, whereby the expression level of target genes is normalized to references (internal controls). Reference genes should be validated, with a stable expression profile

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across all experimental conditions and possible tissues (Bustin et al. 2009). The accuracy and reliability of qPCR is lost if inappropriate reference genes are selected. Therefore, the selection of suitable reference genes is critical when normalizing gene expression data (Bustin et al. 2009, 2010; Jarosova and Kundu 2010). Nevertheless, increasing evidence has indicated that expression of reference genes may vary among species, in different tissue samples, and under different experimental conditions (Czechowski et al. 2005; Hong et al. 2008; Jarosova and Kundu 2010). Variation of expression stability may be due to reference gene products not only driving basic cell metabolism but also affecting other cellular processes (Singh and Green 1993). Thus, the systematic validation of a gene or set of genes with stable gene expression as an internal control must be conducted before any meaningful qPCR analyses are undertaken.

To date, most reference gene expression studies in plants focused on species with sequenced genomes, such as model species and important crop species (Czechowski et al. 2005; Hong et al. 2008; Jarosova and Kundu 2010). The validation of reference genes has received limited attention in weeds, especially in agricultural weeds (Duhoux and Délye 2013; Lee et al. 2010; Petit et al. 2012). To our knowledge, there have been no studies on the suitability of reference genes for qPCR studies on the differential expression of Japanese foxtail genes.

Materials and Methods

Plant Material. Two Japanese foxtail populations, JLGY-1 and JLGY-4, from Ganyu in eastern China were used in this study. JLGY-4 has evolved resistance to the ACCase inhibitor fenoxaprop, and JLGY-1 is sensitive to ACCase inhibitors (Xu et al. 2014). The seeds of JLGY-1 and JLGY-4 were propagated as described in Wu et al. (2016) for use in this study. The seedlings were sown in 12-L pots filled with a 1:1 (w/w) mixture of sand and peat moss and cultured in a greenhouse under 20/15 C day/night temperatures (± 3 C) with a 12/12 h light/dark cycle.

A time-course experiment was conducted at the 3- to 4-leaf stage. Different vegetative tissue samples (root, stem, and leaf) from both populations were separately collected at 0 h (control treatment), 2 h, 6 h, 12 h, 24 h, 3 d, 5 d, and 7 d after herbicide application. The collected samples were immediately frozen in liquid nitrogen and stored at −80 C prior to RNA extraction. Fenoxaprop, a widely used ACCase inhibitor, was applied as the herbicide treatment at the recommended rate (62.0 g ai ha^{-1}). The herbicide application was carried out as described previously (Xu et al. 2013).

A total of 96 samples (two populations times three different tissues times eight treatments times two individual plants as biological replicates) were used in this study. These samples were subdivided into different data subsets: a roots subset (32 root samples from the controls and from those treated with fenoxaprop), a stems subset (32 stem samples from the controls and from those treated with fenoxaprop), and a leaves subset (32 leaf samples from the controls and from those treated with fenoxaprop).

RNA Extraction and Complementary DNA (cDNA) Synthesis. Frozen Japanese foxtail tissues were ground independently in liquid nitrogen. Total RNA was extracted using the RNAsimple Total RNA Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The integrity of the RNA was checked on a 1.5% agarose gel and visualized by ethidium bromide staining. The concentration and purity of the total RNA samples were determined by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Each RNA sample was assayed twice, and the average value was determined. The quality of the total RNA was assessed by the OD260/OD280 and OD260/OD230 ratios. The PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Otsu, Japan) was used to eliminate genomic DNA (gDNA) and synthesize cDNA according to the manufacturer's instructions. Two independent reverse-transcription reactions were conducted to obtain technical replicates for each RNA sample (400 ng).

Selection of Reference Genes and Primer Design. To identify suitable reference genes for Japanese foxtail, we selected eight candidates frequently used as reference genes in other plants. The eight genes were *glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ubiquitin (UBQ), capsine phosphatase (CAP), beta-tubulin (TUB), eukaryotic initiation factor 4a (EIF4A), elongation factor-1 alpha (EF1), 18S ribosomal RNA (18S)*, and *25S ribosomal RNA* (25S) (Table 1).

Very limited genomic data are currently available for Japanese foxtail. Therefore, primers for the reference genes were obtained from blackgrass (*Alopecurus myosuroides* Huds.) (*GAPDH*, *UBQ*, and *25S*) or their design was based on conserved

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Table 1. Candidate reference genes tested and their primer information.

Gene name	Primer sequences (5′ → 3′)	Amplicon length	Amplification efficiency	Correlation coefficient	Average Cq value
		bp	%		
GAPDH	F: GTATTGTTGAGGGACTGATGACC R: AGTAAGCTTGCCATTGAACTCAG	182	110	0.998	22.49
UBQ	F:GCAAGAAGAAGACCTACACCAAG R: CCTTCTGGTTGTAGACGTAGGTG	225	102	0.996	15.70
CAP	F: CTGCGGCTTCTGCTTCGTAC R:CCATTGCCTGCCTTCTTGAA	136	95	0.993	23.14
TUB	F: TACTGTGGTTGAGCCATACAATG R: GTCAGCTTGAGAGTCCTGAA	123	104	0.999	20.10
EIF4A	F: TCTGCTACCATGCCTCCT R: CAGCTTCCACTCTTCCTT	144	104	0.998	19.98
EF1	F: CCGTGACTTCATCAAGAACA R: GGAGATACCAGCCTCAAAAC	100	106	0.999	16.63
18S	F: ATGGCCGTTCTTAGTTGGTG R: TAAGAAGCTAGCTGCGGAGG	110	95	0.993	9.47
25S	F: GCATGAATGGATTAACGAGATTC R:GGCTCCCACTTATCCTACAC	165	104	0.999	9.73

regions in homologous genes of other grasses (Dombrowski and Martin 2009; Duhoux and Délye 2013; Jarosova and Kundu 2010; Lee et al. 2010; Ovesna et al. 2012; Petit et al. 2012; Rechsteiner et al. 2006; Sato et al. 2009; Soderlund et al. 2009; Young et al. 2005). To ensure optimal polymerization specificity and efficiency, the primers were designed using Primer 3 software and selected using melting temperatures between 58 and 62 C, lengths between 18 and 24 bp, GC contents between 45% and 55%, and PCR amplicon lengths between 100 and 225 bp (Table 1) (Rozen and Skaletsky 2000). All primer pairs were initially tested using standard reverse transcription PCR (RT-PCR) to detect their specificity. Amplification of single products of the expected size was tested by electrophoresis on a 2% agarose gel. The amplicons were cloned and sequenced on both strands to confirm that the targeted gene had been amplified (Supplemental Table S1). Furthermore, to rule out any gDNA contamination, the primers targeting *GAPDH* were used to amplify an intron-containing amplicon as described by Petit et al. (2012).

qPCR. The qPCR was conducted on a 7500 Real-Time PCR Detection System (Applied Biosystems, Foster City, USA) using the SYBR Premix Ex Taq (TaKaRa) and following the manufacturer's instructions. The reactions were performed in a total volume of 20 µl containing 2 µl of 10-fold diluted cDNA template, 0.8 µl of each primer (10 µM), 0.4 µl of ROX reference dye II, and 10 µl of SYBR Premix Ex Taq. The qPCR was conducted on a 96-well reaction plate using the parameters recommended by the manufacturer (95 C for 30 s followed by 40 cycles of 95 C for 5 s and 60 C for 34 s). The specificity of the amplicons was verified by melting curve analysis (60 to 95 C) and by agarose gel electrophoresis. For each primer pair, no template controls were also performed. Every diluted sample was amplified twice in two independent qPCR runs for each gene. gDNA contamination was checked by searching for the amplicons of *GAPDH*, which flank an intron-containing region. The amplification efficiencies (E) and correlation coefficients of the PCR amplifications were derived from a standard curve generated by a 5-fold serial dilution points of cDNA combined with a mix containing the samples from the controls.

Data Analysis. The expression levels of the candidate reference genes were determined by calculating the quantification cycle (Cq) values, where the quantification of the fluorescence reached a specific threshold level of detection. Three different and widely used reference gene–screening software programs—geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), and BestKeeper (Pfaffl et al. 2004)—were used to rank the expression stability of reference genes across all of the experimental sets. The Cq values were calculated by the SDS software in ABI 7500 v.1.4. For geNorm and NormFinder, the input data are supposed to be on a linear scale. Therefore, the Cq values were converted to relative quantities using the formula: $E^{-\Delta Cq}$ before calculation (Livak and Schmittgen 2001). Relative quantities were

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used for geNorm and NormFinder, but the untransformed Cq values were used for BestKeeper.

The geNorm software provides an expression stability (M) value and excludes the least stable genes using a stepwise method. It also creates a line graph to show the expression stability of the selected genes. Genes with the lowest M values express most stably and therefore would be selected as the most suitable reference genes (Vandesompele et al. 2002). An M value below 1.5 shows that the reference genes have stable expression (Vandesompele et al. 2002). As a single reference gene is rarely adequate for normalization, geNorm also estimates the number of genes required to calculate the number of factors needed for robust normalization and performs a stepwise analysis to calculate the pairwise variation (Vn/Vn + 1) between two sequential normalization factors that contain increasing numbers of genes (Vandesompele et al. 2002).

NormFinder estimates within and among groups based on ANOVA model variation and ranks the reference genes according to the stability value (SV) of their expression patterns under certain experimental conditions (Andersen et al. 2004). Reference genes with the lowest SVs are considered the ideal reference genes.

BestKeeper estimates gene expression stability for all individual reference genes based on the SDs calculated from the Cq values. A low SD (<1) for the Cq value is necessary for reference genes to be useful (Pfaffl et al. 2004). The remaining genes were each ranked based on pairwise correlations and the geometric mean of the Cq values. Candidate genes with the strongest correlations with the BestKeeper index are considered to have the most stable gene expression.

Results and Discussion

Efficiency of the Candidate Reference Genes and qPCR Specificity. A total of eight candidate reference genes were assessed using qPCR (Table 1). The presence of a single peak in the qPCR melt curve products (Supplemental Figure S1) and a single band in the gel electrophoresis results indicated that there were no primer dimers or other nonspecific amplification products. The absence of gDNA contamination was checked by the single-peak melting curves of the targeted *GAPDH*. The sequence correctness of the eight candidate reference genes was proven by standard RT-PCR, and the obtained sequences are listed in Supplemental Table S1. In addition, no qPCR detection signals were seen in the PCRs that did not have a template.

The amplification efficiency and correlation coefficients are given in Table 1. The amplification efficiency ranged from 95% to 110%, and the coefficients varied from 0.993 to 0.999 (Table 1), which confirmed that the qPCR systems used in this study were acceptable and comparable.

Expression Levels of the Candidate Reference Genes. To give an overview of the transcript levels of the eight candidate reference genes, we determined their expression based on Cq values in all the samples and created box-and-whisker plots (Figure 1). The mean values of the reference genes were between 9.47 and 23.14, which represented the different expression levels in Japanese foxtail (Figure 1). *CAP* had the lowest expression level, with a mean Cq value of 23.14 cycles. In comparison, the Cq values of *18S* and *25S* reached only 9.47 and 9.73 cycles, respectively, which indicated high levels of expression compared with the other reference genes. Generally, the expression levels of most genes ranged from 15.70 to 23.14. While *18S* showed stable gene expression, *TUB* expression was very variable (Figure 1). Japanese foxtail showed wide ranges of expression variation and abundance with respect to these candidate reference genes, which indicated that no single gene had a stable and constant expression level under the sets of conditions evaluated here. Therefore, it was necessary to screen appropriate reference genes via statistical methods in the analyses.

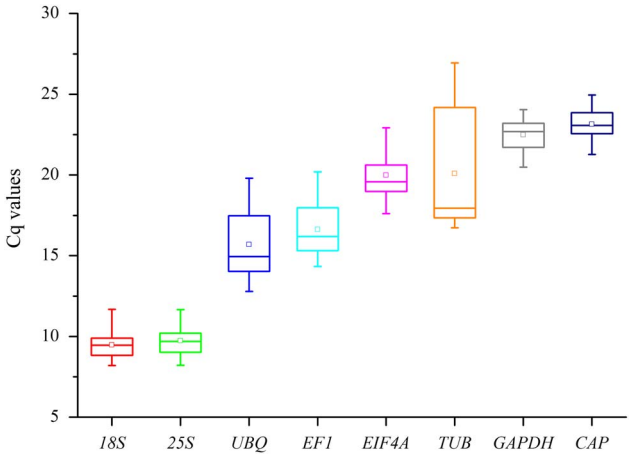

Figure 1. Quantification cycle (Cq) values of the candidate reference genes in the Japanese foxtail samples. The top and bottom borders represent the 25th and 75th percentiles. The horizontal line within the box indicates the median. The small inner box indicates the mean. Whiskers represent the maximum and minimum values.

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Expression Stability of Candidate Reference Genes. In plant molecular biological research, qPCR has emerged as a powerful and important tool for studying gene expression (Bustin 2002). However, for valid qRT-PCR analysis, the expression pattern should be normalized using stably expressed reference genes. Thus, the systematic validation of suitable reference genes for specific experimental conditions and species is extremely important when using qPCR (Fu et al. 2013; Gutierrez et al. 2008). Additionally, the systematic validation of suitable reference genes may be more important when differences in expression of target genes are small. A number of studies have indicated that optimum reference genes can even vary among different tissue samples (Brunner et al. 2004; Jarosova and Kundu 2010; Jiang et al. 2014; Kong et al. 2014; Lee et al. 2010; Yang et al. 2014). Therefore, we investigated the expression levels of eight genes in different tissues (root, stem, and leaf). Validation of reference gene expression stabilities requires mathematical methods. Fortunately, some algorithms, such as geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and the ΔCt approach (Silver et al. 2006) have been developed to simplify the validation. To obtain more reliable reference genes for normalization, we used three algorithms: geNorm, NormFinder, and BestKeeper to evaluate reference gene expression stability in Japanese foxtail.

The results obtained using geNorm are shown in Figure 2. When all 96 Japanese foxtail samples were considered together, all the genes, except *TUB* ($M > 1.5$), were found to be suitable for normalization (Figure 2A). The average expression M values for *18S* and *25S* were the lowest, and the value for *TUB* was the highest (Figure 2A). This suggests that the *18S* and *25S* expressions were the most stable and *TUB* was the least stable. In the roots subset, *UBQ* and *TUB* performed well, with an M value of 0.393, and *GAPDH* had a high M value (Figure 2B). *UBQ* and *EF1* were the most stable genes, both with an M value of 0.512, and *GAPDH* was the least stable gene in the stems subset (Figure 2C). Both *18S* and *25S* were stably expressed in the leaves subset, and *TUB* was the least stable (Figure 2D).

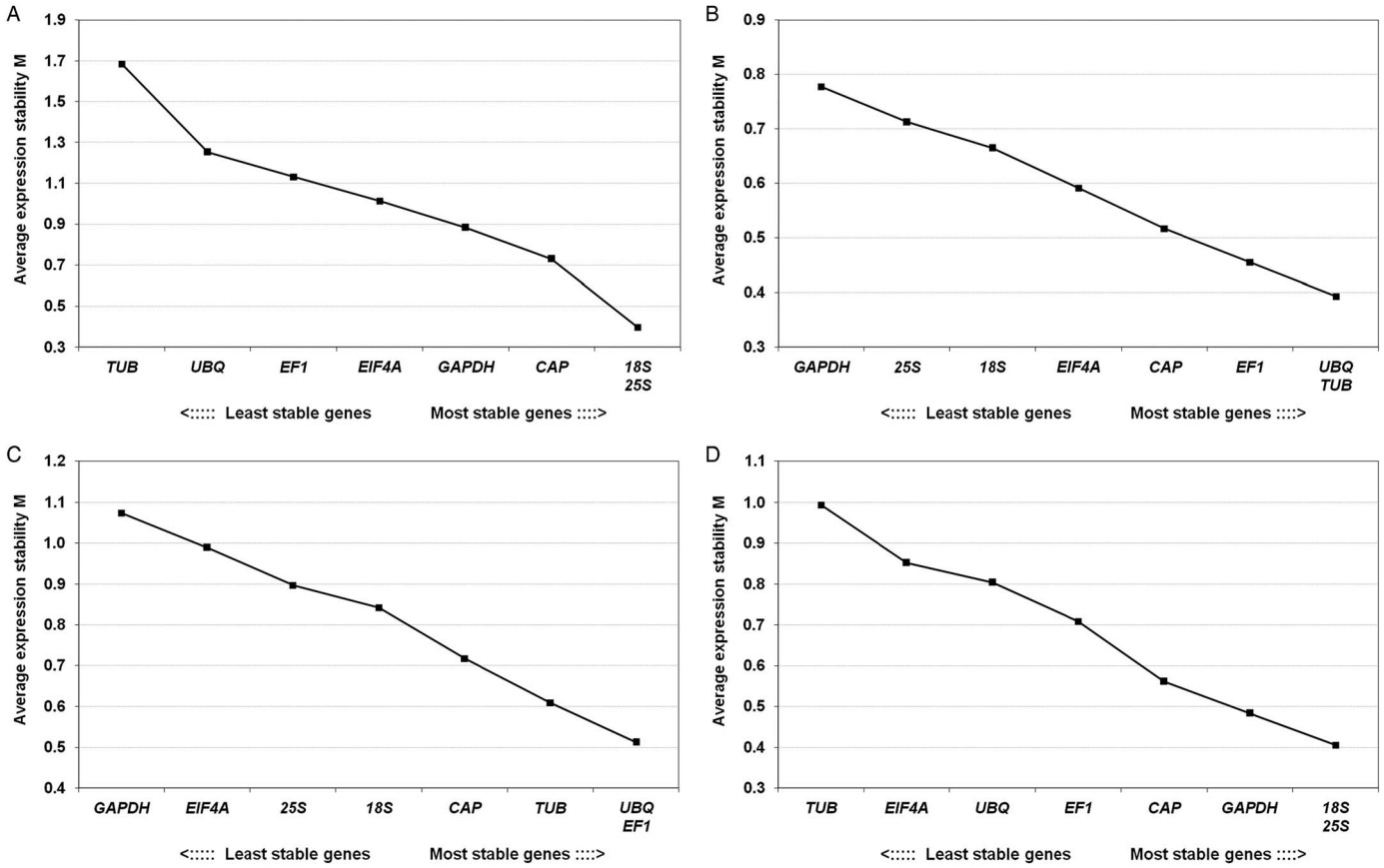

Figure 2. Expression stability and ranking of candidate reference genes as calculated by geNorm. Average expression stability of the reference genes was measured during stepwise exclusion of the least stable reference genes. (A) All samples, (B) roots subset, (C) stems subset, and (D) leaves subset.

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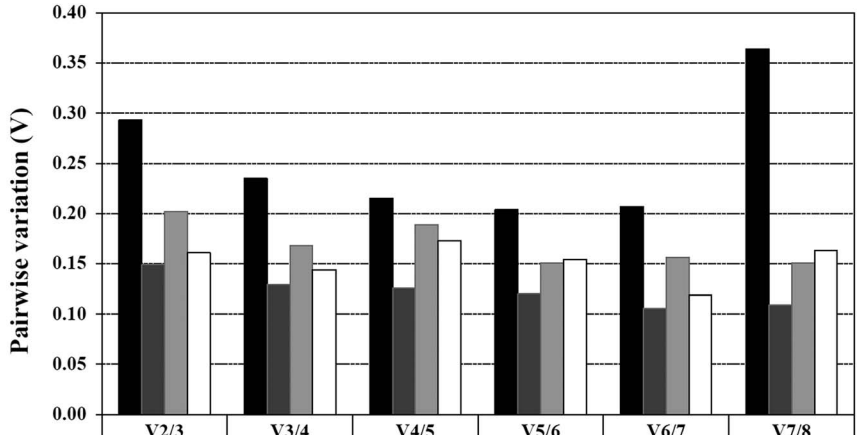
The use of two or more reference genes for qPCR studies is generally agreed to generate more reliable results. Therefore, pairwise variations for each data subset were calculated using geNorm so that the optimal number of internal control genes for normalization could be determined. The original publication describing geNorm (Vandesompele et al. 2002) proposed that a value below a threshold of 0.15 for the pairwise variation meant that the inclusion of an additional reference gene was unnecessary. Based on the 0.15 threshold, two genes were needed for the roots subset and three in the leaves subset (Figure 3). Nevertheless, 0.15 is not an absolute cutoff value, but rather an ideal value, which is dependent on the expression of the genes and the diversity of the samples tested (Duhoux and Délye 2013; Fu et al. 2013; Petit et al. 2012; Xu et al. 2012). Figure 3 gives guidance for determining the optimal number of reference genes. In this study, pairwise variations were above 0.15 for the stems subset and when the all samples were pooled together. We chose four reference genes to normalize gene expression in the stems subset, as the inclusion of the five genes had an insignificant effect on the pairwise variation (Figure 3). However, we failed to calculate the optimal number of reference genes when all the samples were combined.

Different algorithm methods sometimes produce different results for the same data set. Hence, all of the data were reassessed by NormFinder to avoid introducing unnecessary bias. The results obtained from NormFinder are summarized in Table 2. Based on the results of NormFinder, the three most stable genes were *EF1*, *UBQ*, and *TUB* in the roots subset; *CAP*, *18S*, and *EF1* in the stems subset; *18S*, *GAPDH*, and *EF1* in the leaves subset; and *CAP*, *EF1*, and *EIF4A* in the different tissues subset. The least stable genes were *GAPDH* in the roots and stems subsets and *TUB* in the other subsets. When all the samples were included, *EF1* (0.277) was identified as the most stable gene, followed by *EIF4A* (0.390) and *CAP* (0.487). *TUB* was least stable (2.007) (Table 2). NormFinder also has the ability to compute the variation between sample groups or treatments, which could be used to determine the best combination of two reference genes for normalization. Thus, we divided the all-samples data set into phenotype groups (resistant or sensitive to fenoxaprop) and tissue groups (root, stem, and leaf) and then analyzed the grouped data using NormFinder (Table 2). Including the group assignment did not affect the ranking of the genes in this analysis, although the stability values were reduced when the group categories were introduced (Table 2). However, the best combination of two genes, *EF1*/*EIF4A* for phenotype groups and *CAP*/*UBQ* for tissue groups, further reduced the NormFinder SVs (Table 2).

BestKeeper was also used for reference gene expression analysis in this study. Table 3 shows the results of the evaluation of candidate reference genes using BestKeeper. In the all-samples data set, *EF1*, *UBQ*, *EIF4A*, and *TUB* were excluded because of their high expression deviations (SD > 1) (Table 3).



	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
All samples	0.293	0.235	0.215	0.204	0.207	0.364
Roots subset	0.149	0.129	0.126	0.120	0.106	0.109
Stems subset	0.202	0.168	0.189	0.151	0.156	0.151
Leaves subset	0.161	0.144	0.173	0.154	0.119	0.163

Figure 3. Pairwise variation to determine the optimal number of reference genes for normalization. The optimal number of genes was determined separately for the roots subset, stems subset, leaves subset, and for all samples combined. The recommended cutoff value under which there is no need for inclusion of another gene is 0.15.

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Table 2. Candidate reference genes' ranking and their expression stability values (SV) as calculated by NormFinder.

	All samples											
	No groups		Phenotype groups		Tissue groups		Roots subset		Stems subset		Leaves subset	
Rank	Gene	SV	Gene	SV	Gene	SV	Gene	SV	Gene	SV	Gene	SV
1	*EF1*	0.277	*EF1*	0.051	*EIF4A*	0.282	*EF1*	0.177	*CAP*	0.232	*18S*	0.324
2	*EIF4A*	0.390	*EIF4A*	0.073	*EF1*	0.309	*UBQ*	0.211	*18S*	0.327	*GAPDH*	0.331
3	*CAP*	0.487	*CAP*	0.101	*CAP*	0.496	*TUB*	0.284	*EF1*	0.470	*EF1*	0.362
4	*UBQ*	0.673	*UBQ*	0.136	*UBQ*	0.583	*CAP*	0.374	*TUB*	0.502	*CAP*	0.425
5	*18S*	0.680	*18S*	0.138	*18S*	0.657	*EIF4A*	0.379	*25S*	0.504	*EIF4A*	0.468
6	*25S*	0.829	*25S*	0.170	*25S*	0.759	*18S*	0.425	*EIF4A*	0.619	*25S*	0.543
7	*GAPDH*	0.930	*GAPDH*	0.190	*GAPDH*	0.792	*25S*	0.547	*UBQ*	0.730	*UBQ*	0.546
8	*TUB*	2.007	*TUB*	0.413	*TUB*	1.716	*GAPDH*	0.590	*GAPDH*	0.813	*TUB*	0.888
Best two genes			*EF1/ EIF4A*	0.045	*CAP/ UBQ*	0.137						

Of the remaining four genes, the highest r (Pearson's coefficient of correlation) values were observed for *CAP*, *EIF4A*, and *TUB* in the leaves subset; *EF1*, *UBQ*, and *TUB* in the different tissues subset were excluded based on their SD values (Table 3). The most stable genes varied in the different tissues. They were *EF1* in the roots subset, *TUB* in the stems subset, and *18S* in the leaves subset. High r values were observed for *CAP*, *18S*, and *EIF4A*, which indicated that these genes could be used to normalize the different tissues subset data (Table 3).

Choice of Suitable Reference Genes. In the roots subset, *EF1* and *UBQ* were always among the four most stable genes for all three algorithms (Figure 2B; Tables 2 and 3). Furthermore, geNorm indicated that the two genes would be best for normalization (Figure 3). Therefore, we selected *EF1* and *UBQ* as the reference gene set suitable for normalization in the root samples from Japanese foxtail subjected to fenoxaprop stress. The results yielded by geNorm, NormFinder, and BestKeeper were similar for the stems subset (Figure 2C; Tables 2 and 3). The number of reference genes required for normalization computed by geNorm was four (Figure 3). Although *UBQ* was ranked as the most stable gene in the stems subset by geNorm, this gene was ranked as the second least stable gene by NormFinder. Thus, *UBQ* was not included in the reference gene set for stems, and we recommend using *EF1*, *TUB*, *CAP*, and *18S* as reference genes for normalization in stems. Analysis using NormFinder and BestKeeper showed that the most reliable reference genes for normalization in the leaves subset were consistent, and *18S*, *GAPDH*, and *EF1* were ranked as the three most stable genes (Tables 2 and 3). While *25S* was given top rank by geNorm, it was ranked sixth by NormFinder and BestKeeper. As three genes were indicated by geNorm (Figure 3) as the minimum number needed, we finally selected *18S*, *GAPDH*, and *EF1* as suitable reference genes for leaves. Overall, different plant tissues had their own best reference genes in Japanese foxtail. This was in accordance with results for

Table 3. Candidate reference genes' ranking according to their stability as calculated by BestKeeper.[a]

	All samples			Roots subset			Stems subset			Leaves subset		
Rank	Gene	r[a]	SD[b]	Gene	r	SD	Gene	r	SD	Gene	r	SD
1	*CAP*	0.817	0.73	*EF1*	0.850	0.51	*TUB*	0.760	0.78	*18S*	0.877	0.54
2	*18S*	0.754	0.63	*18S*	0.772	0.55	*CAP*	0.743	0.55	*EF1*	0.867	0.81
3	*25S*	0.605	0.63	*25S*	0.747	0.64	*EF1*	0.724	0.71	*GAPDH*	0.851	0.58
4	*GAPDH*	0.411	0.75	*UBQ*	0.703	0.40	*18S*	0.669	0.45	*UBQ*	0.787	1.00
5	*EF1*	0.954	1.38	*TUB*	0.614	0.34	*UBQ*	0.615	0.85	*CAP*	0.703	0.40
6	*UBQ*	0.931	1.77	*EIF4A*	0.427	0.77	*25S*	0.580	0.59	*25S*	0.685	0.59
7	*EIF4A*	0.857	1.11	*CAP*	0.373	0.46	*EIF4A*	0.031	0.62	*EIF4A*	0.860	1.02
8	*TUB*	0.937	3.27	*GAPDH*	0.309	0.45	*GAPDH*	0.015	0.78	*TUB*	0.763	1.18

[a] r, Pearson's coefficient of correlation. SD, SD of the quantification cycle values; genes with SD values higher than the threshold value (1.00) are underlined.

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perennial ryegrass (*Lolium perenne* L.) (Lee et al. 2010) and melon (*Cucumis melo* L.) (Kong et al. 2014), which also showed that the best reference genes varied depending on the plant tissue to be analyzed. These results indicated that reference genes vary depending on the experimental conditions, and systematic validation of suitable reference genes must be conducted before any meaningful qPCR analyses can be carried out.

When the complete data set was analyzed, the results produced by geNorm and BestKeeper were similar, but they differed from the results calculated by NormFinder (Figure 2A; Tables 2 and 3). Studies using these algorithms have shown that they may produce minor differences in gene stability rankings (Duhoux and Délye 2013; Hong et al. 2008; Jarosova and Kundu 2010; Petit et al. 2012; Yang et al. 2014) or relatively substantial differences (Jiang et al. 2014; Lee et al. 2010; Tong et al. 2009). It is not surprising that the ranking of candidate reference genes by geNorm, NormFinder, and BestKeeper were not always identical, as these algorithms rely on different mathematical approaches to calculate stability (Andersen et al. 2004; Pfaffl et al. 2004; Vandesompele et al. 2002). For example, geNorm is mainly dependent on the assumption that none of the reference genes being analyzed are coregulated, as this would lead to inaccurate results (Andersen et al. 2004). Furthermore, geNorm only compares the expression ratio of different genes, regardless of the high variation within a given reference gene. This may also lead to inappropriate conclusions (Lin and Lai 2010). BestKeeper was limited to the heterogeneous variance between groups of differentially expressed genes (Pfaffl et al. 2004). In the all-samples data set, geNorm and BestKeeper were not appropriate for normalizing the highly variable expressions of the reference genes we studied (average Cq values from 9.47 to 23.14) (Figure 1). Thus, we recommend the use of *EIF4A*/*EF1* or *UBQ*/*CAP* as suitable reference genes for normalizing gene expression in Japanese foxtail, as indicated by NormFinder when all the samples were analyzed together. NormFinder was also chosen as the appropriate algorithm when the reference genes tested produced significantly variable results during a qPCR study of the longan tree (*Dimocarpus longan* Lour.) (Lin and Lai 2010).

The Reference Genes Identified Were Helpful in Studying Resistance Mechanisms. Although increased expression of the target protein has been shown to confer resistance to some herbicides in weeds (e.g., glyphosate resistance in Palmer amaranth (*Amaranthus palmeri* S. Wats.), it has rarely been confirmed in ACCase inhibitor–resistant weeds (Bradley et al. 2001; Délye 2005; Délye et al. 2013; Powles and Yu 2010). As far as we know, only a few studies have attempted to investigate the expression levels of *ACCase* (Bradley et al. 2001; Cha et al. 2014; Duhoux and Délye 2013; Petit et al. 2012). In blackgrass, *ACCase* had a similar expression level in plants that are either sensitive or resistant to the ACCase inhibitor fenoxaprop (Petit et al. 2012). In *Lolium* spp. the *ACCase* expression levels did not vary between sensitive and resistant plants subjected to acetolactate synthase inhibitors, which do not target ACCase (Duhoux and Délye 2013). However, Bradley et al. (2001) determined that the resistance to quizalofop-P and sethoxydim was conferred by an overproduction of ACCase in johnsongrass [*Sorghum halepense* (L.) Pers.]. Cha et al. (2014) found the *ACCase* expression was up-regulated at 3 d after fluazifop treatment in goosegrass [*Eleusine indica* (L.) Gaertn.]. The overexpression of *ACCase* may be caused by gene amplification, changes in a gene promoter, or other changes in gene regulation (Cha et al. 2014; Powles and Yu 2010). Although this has not been found in ACCase inhibitor–resistant weeds, gene amplification–conferred resistance has been documented in other herbicide-resistant weeds. Gaines et al. (2010) found that a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene amplification in resistant Palmer amaranth resulted in high *EPSPS* expression levels, and this mechanism imparted high-levels of glyphosate resistance.

The reference genes identified in this study will be of great help in the study of resistance mechanisms and other molecular regulation mechanisms subjected to herbicide stress in Japanese foxtail. NTSR is the main cause of herbicide resistance in grass weed species and is a complex polygenic adaptation to herbicides that remains to be studied (Délye et al. 2013). So far, only TSR has been investigated in Japanese foxtail. NTSR is considered to be endowed by differential regulation of many stress-responsive genes in resistant plants compared with sensitive plants (Délye 2005; Délye et al. 2013). Thus, the reference genes identified in this study will be key to the study of the genes governing NTSR in Japanese foxtail. Recently, novel high-throughput sequencing technologies (e.g., RNA-seq) have offered new approaches to transcriptome profiling. RNA-seq enables researchers to compare the full

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transcriptomes of resistant and sensitive plants, which will help scientists to decipher potential resistance mechanisms in plants. However, the RNA-seq data should be further confirmed by qPCR. Our reference genes should therefore be used during RNA-seq analysis.

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Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2017.19

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