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Original Research

Validation of expression stability of reference genes in response to herbicide stress in wild oat (*Avena ludoviciana*)

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Abstract: Weeds are serious problem in crop production and wild oat is a grass weed of economic and agronomic significance. We need to extend our basic knowledge of weeds especially in molecular genetics and gene expression. For study of gene expression by semi-quantitative and quantitative PCR, it is recommended that normalization of reference genes be carried out in order to select the most stable reference gene for a precise gene expression study. The purpose of this research was evaluation of four reference genes in response to treated and untreated (control) by herbicide in two tissues (stem and leaf) of non-target site resistance wild oat (*A. ludoviciana*). Four candidate reference genes including Actin, Ef1α (elongation factor 1 alpha), GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and TBP (TATA-box-binding protein) were used to determine stable reference gene exposed to the herbicide using the statistical methods of NormFinder, BestKeeper and delta-Ct. NormFinder indicated that TBP and Actin genes are the best combination of two genes for normalizing calculations (with a combined gene stability value of 0.012) for qPCR analysis under herbicide stress in different tissues of non-target site resistance wild oat. Based on the statistical results, the Ef1α gene was identified as the unstable reference gene. Totally, according to results of this study, TBP gene is the most stable reference gene and therefore, this gene can be used as a reference gene for future studies of quantitative PCR analysis of herbicide stress-responsive gene expression in wild oat and potentially in other grass weed species.

Key words: Gene stability; Herbicide resistance; Tissue; Real-time PCR; Wild oat.

Introduction

Weeds response to environmental stresses is mediated by the regulation of gene expression. A major abiotic stress faced by arable weeds infesting agricultural fields is herbicide applications. Therefore, herbicide applications trigger stress response pathways in weed plants (1) and we require to study molecular mechanisms behind this phenomena. Recently, the techniques of molecular biology provide an approach of biological research and have provided wonderful insights into the biology of weeds, including their origin, dispersal, and mechanisms of control (2, 3). Gene expression pattern is indicative of the tendency to gene expression regulation and provides a novel insight for understanding the biological functions of genes. Analysis of gene expression is increasingly important in biological studies and the qPCR technique is becoming the selected method for high-throughput and precise expression profiling of selected genes. The qPCR is one of the most common techniques used to study of gene expression (4). This is a quick and sensitive technique, enables users to quantitative analysis (5). Analyses of qPCR require the normalization of expression data using a reference gene(s) that transcription level of this gene is stable in all cells and is not influenced by experimental or environmental agents (6).

Normalizing the expression data of the target genes against those of the housekeeping genes (reference gene) enables the determination of whether the target gene expression levels are up or down-regulated. Although the expression levels of reference genes are considered stable, they may actually vary under different conditions (7). For example, expression levels of the glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) increased 30-fold in tomato (*Solanum lycopersicum* L.) plants under cold stress. Thus, this gene should not be used as a reference gene for *S. lycopersicum* gene expression analyses involving temperature changes (8). A small number of reference genes have been approved for weeds because most weeds are species without associated genomic resources. Several reports have shown that the expression of many widely used reference genes can vary considerably with the experimental conditions, tissues and species (9, 10). As a consequence, no "universal" reference gene has been identified to date (11).

Herbicides are one of the most important elements in control of weeds in modern agriculture. Because of the repeated use of herbicides, the herbicide-resistant weed populations have evolved around the world (12). Herbicide resistance is an evolutionary process and a classic example of rapid dynamic adaptation to human-mediated selection pressure (13). This evolutionary process largely depends on the biology of the weed species, the biochemical properties of herbicides, the number and rate of herbicide application, management factors, and genetics such as the frequency of resistant alleles and their associated fitness cost (1, 14). There are two

basic mechanisms regulating herbicide resistance. First, target-site resistance is associated with point mutations in target enzyme coding sequences or it is a result of enzyme overexpression. Second, non-target-site resistance involves enhanced herbicide metabolism, reduced translocation, or sequestration. In general, non-target-site resistance is more common. Group A (i.e. ACCase) herbicides have been widely used for wild oat control. Resistance to ACCase herbicides in wild oats was first identified in a population collected from York in Western Australia in 1985. This population was confirmed to be resistant to diclofop-methyl in pot trials (15).

Designing a reliable qPCR analysis is a requirement for the proper validation of gene expression data. Quantification of the expression of such genes requires normalizing qPCR data using reference genes with stable expression in the system studied as internal standards. A reference gene must be constitutively and constantly expressed in all experimental conditions and samples studied. In this study, we have performed qPCR experiments to determine the most stable reference gene to study of quantitative expression based on the reference genes of Actin, GAPDH, TBP and Ef1α. The expression patterns of these reference genes were tested in the different tissues (stem and leaf) and under herbicide application to identify the most suitable gene(s) for use as internal controls in qPCR expression studies in wild oat and potentially for other *Avena* species.

Materials and Methods

Herbicide treatment

In this study, tolerance and sensitivity responses among wild oat biotypes were performed in response to ACCase-inhibiting (clodinafop propargyl herbicide) in a greenhouse. The clodinafop propargyl herbicide was used at 256 g a.i. ha^{-1} and spraying was carried out at the growth stage 3-4 leaves. Expression of non-target-site-based resistance (NTSR) genes enables resistant plants to survive in herbicide exposure (16, 17). Therefore, in current study three NTSR biotypes to ACCase-inhibiting herbicides collected from fields of Kermanshah province (west of Iran) were investigated. These biotypes were examined under two different conditions (treated and untreated by herbicide) and the samples were collected in 24 hours after herbicide treatment. A total of 24 samples including three biotypes, two different tissues (stem and leaves), two different treatments (with herbicide) and two individual plants (as biological replicates) were used to study of gene expression analysis. Different tissue samples from treated and untreated were harvested and then stored in the liquid nitrogen for a survey of gene expression.

Primer design

The candidate reference genes selected for this study were those that their expression had been demonstrated stable under various stresses in *Lolium sp.* (11, 18, 19) or in other grasses (20-22). Four reference genes including Actin, Ef1α (elongation factor 1 alpha), GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and TBP (TATA-box-binding protein) were used to determine stable reference gene exposed to the herbicide. Due to the unknown sequence of the genes studied in the wild oat, the nucleotide sequences of the genes studied from oat family plants were obtained from the NCBI database. The sequences were aligned against ESTs of *A. sativa*. The EST sequences were matched with high similarity, assembled with VectorNTI software. Primers were designed using AlleleID software, which is specific for real-time (Table 1).

RNA isolation

Total RNA was extracted from leaf and stem tissues collected pre-treatment and 24 hours after treatment using an RNX-plus buffer (SinaClon BioScience, Tehran, Iran) according to the manufacturer's instructions. RNA quantification was done using the Thermo scientific Nanodrop™ 2000c spectrophotometer (Wilmington, USA). Also, all RNA isolation had an OD 260/280 between 1.8 and 2.0. Elimination of genomic DNA from RNA was performed with DNaseI, RNase-free (Fermentase, Germany). The RNA integrity was assessed by 1% agarose gel electrophoresis through visualization of the two ribosomal subunits (28S and 18S).

Quantitative PCR

The cDNA synthesis was performed for each sample using the oligo-dT primer, random hexamer primer and M-MuLV reverse transcriptase. Purified RNA (2 µg) was mixed with oligo-dT (100 µM) and random hexamer (100 µM) primers (Thermo Fisher Scientific, USA), then nuclease-free water was added up to the volume of 12 µL. Immediately heated at 65°C for 5 min and rapidly cooled on ice. The samples were then added to a reverse transcription mix containing, buffer M-Mulv (5X), M-Mul V reverse transcriptase (200 u/µl), RiboLock RNase inhibitor and dNTP mix (10 mM). Samples were incubated at 25°C for 5 min, and then at 42°C for 60 min. The cDNA samples (20 µL) were diluted with 10 µL DNase-free water and used for qPCR. Each experiment was done by two biological replicates

Table 1. Primer sequences for qPCR analysis of candidate reference genes.

Reference gene	Primer sequences (5′-3′)	Amplicon length (bp)	PCR efficiency (%)	Annealing temperature (°C)
Actin	F: GTAACATTGTGCTCAGTGGTG R: TACTTCCTCTCGGGTGGTG	127	91	54.6
Ef1α	F: CAAGAATGTTGCCGTGAAGG R: GCCGTTGCCAATCTGACC	133	94	55.9
GAPDH	F: TTGATCTCACCGTCAGAATCG R: TGCTGTCGCCAATGAAGTC	144	118	54.6
TBP	F: ATGGTGCTTTCTCAAGTTATG R: CGAAGGCAGTGTATGTCTC	147	112	51.7

F: forward primer; R: reverse primer

and two technical replicates. The real-time PCR amplification was carried out using in a 20 µL amplification mixtures containing 10 µL 2×SYBR Green premix Ex Taq™ (TaKaRa, Dalian, China), 3 µl of diluted cDNA (as template), 0.8 µl of each primers (10 µM) and 5.4 µL nuclease- free water. Reactions were run on the Rotor-Gene Q (Qiagen, Germany). The PCR program consisted of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec, an annealing step for 15 sec (temperatures listed in Table 1), and 72°C for 15 sec. Statistical significance was calculated by Student's t-test.

Normalization of reference genes

The selection of the best endogenous reference genes for this study was performed using NormFinder (23), BestKeeper (24) and comparative delta-Ct (25). NormFinder was used to determine the stability of reference genes based on inter- and intra-group variance in expression level. The stability value (SV) was calculated, with the lower stability value indicating the higher stability (23). Using BestKeeper, variations in the cycle threshold (Ct) and standard deviation (SD) based on Ct values were calculated. Genes with SD>1 were considered unstable and eliminated. The comparative delta-Ct method examined the relative expression between gene pairs. The reference genes were ranked or discarded depending on the Ct values for analyzed genes (25).

Results

Performance analysis of qPCR primers

The melting results obtained from the quantitative analysis indicated that primer-dimer and the extra band were not observed and was formed the single peak graph (Figure 1). Agarose gel electrophoresis and single-peak melting curves confirmed the absence of primer-dimer and non-specific amplification for studied genes. The PCR efficiency ranges from 91% (Actin) to 118% (GAPDH) (Table 1).

Expression levels of the candidate reference genes

To give an overview of the transcript levels of the four candidate reference genes, we determined their expression based on Ct values in all the samples. The mean values of the reference genes were between 8.77 and 17.97 for TBP and Ef1α, respectively, which represented the different expression levels in wild oat (Figure 2). The three GAPDH, Ef1α, and Actin genes did not show significant differences in Ct values, while these genes showed a significant difference in Ct values with TBP gene. Ef1α had the lowest expression level with a mean Ct value of 17.97 cycles. In comparison, the Ct value of TBP reached only 8.77 cycles, which indicated high levels of expression compared with the other reference genes. Wild oat 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.

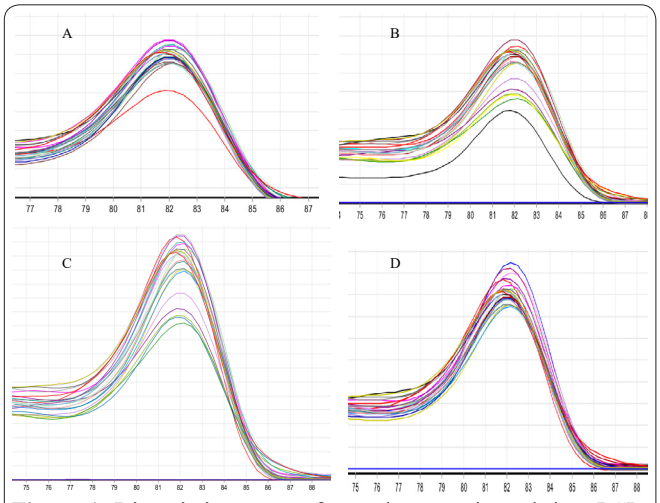

Figure 1. Dissociation curves for study genes in real-time PCR. (A) Actin, (B) Ef1α, (C) GAPDH and (D) TBP.

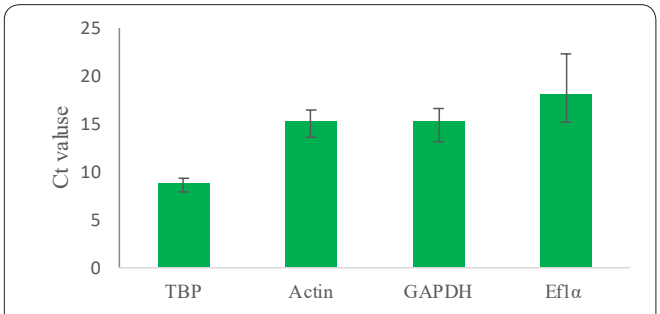

Figure 2. Cycle threshold (Ct) values of the candidate reference genes in the wild oat samples. Vertical columns represent the average and error bar indicates the maximum and minimum Ct values.

BestKeeper analysis

By the BestKeeper software, compared with Actin, TBP and GAPDH observed, Ef1α was a high level of Ct variation, which it is a reason to exclude Ef1α from index calculation. In our BestKeeper analysis to recognize the best reference gene showed that the TBP was the most stable gene in comparison with other reference genes for qPCR analysis in the different tissues under untreated or treated with herbicide. Expression stability of Actin was higher than GAPDH and Ef1α reference genes (Figure 3). Standard deviation (SD) of TBP, Actin and GAPDH genes were less than one (SD <1), but the Ef1α gene showed a standard deviation greater than one (SD >1). Compared with the TBP, gene expression of Ef1α showed the most Ct variations (SD=1.03). Also, the Ef1α gene showed the highest standard deviation of the absolute regulation coefficients (±2.04). While the TBP gene showed the least amount of Ct variations (SD=0.32) and the lowest SD of the absolute regulation coefficients (±1.24), the lowest expression regulation, indicating the sustainability of the expression of the gene in the herbicide and non-herbicide treatments for wild oat plants (Table 2).

The results of ranking based on BestKeeper software showed that TBP gene was recognized as the most stable reference gene under different conditions of herbicide treatment and in different tissues in wild oat. While the Ef1α gene in herbicide treatment conditions and GAPDH gene in various tissues was recognized as the most unstable reference gene (Table 3).

Table 2. Descriptive statistics of reference genes using BestKeeper software.

Factor	TBP	Actin	GAPDH	Ef1α
N	24	24	24	24
GM [Ct]	8.77	15.18	15.13	17.92
AM [Ct]	8.77	15.21	15.16	17.98
Min [Ct]	7.93	13.54	13.12	15.20
Max [Ct]	9.21	16.45	16.54	22.30
SD [±Ct]	0.32	0.79	0.85	1.03
CV [%Ct]	3.59	5.18	5.59	5.71
Min [x-fold]	-1.79	-3.12	-4.02	-6.61
Max [x-fold]	1.36	2.41	2.66	20.76
SD [± x-fold]	1.24	1.73	1.80	2.04

Abbreviations: N: number of samples; GM [Ct]: the geometric mean of Ct; AM [Ct]: the arithmetic mean of Ct; Min [Ct] and Max [Ct]: the extreme values of Ct; SD [± Ct]: the standard deviation of the Ct; CV [%Ct]: the coefficient of variance expressed as a percentage on the Ct level; Min [x-fold] and Max [x-fold]: the extreme values of expression levels expressed as an absolute x-fold over- or under-regulation coefficient; SD [± x-fold]: standard deviation of the absolute regulation coefficients.

Table 3. Ranking of the candidate reference genes according to their stability value using BestKeeper and NormFinder.

Reference gene	BestKeeper						NormFinder					
	All samples		Herbicide effect (Untreated and treated)		Tissue groups (leaf and stem)		All samples		Herbicide effect (Untreated and treated)		Tissue groups (leaf and stem)	
	SD	Rank	SD	Rank	SD	Rank	SV	Rank	SV	Rank	SV	Rank
TBP	0.32	1	0.19	1	0.11	1	0.149	1	0.08	1	0.17	1
Actin	0.79	2	0.44	2	0.26	3	0.175	2	0.27	3	0.23	2
GAPDH	0.85	3	0.41	3	0.32	4	0.347	3	0.12	2	0.47	4
Ef1α	1.03	4	0.67	4	0.23	2	0.631	4	0.41	4	0.31	3

Best combination of two reference genes by NormFinder: TBP and Actin= 0.012.

NormFinder analysis

Based on NormFinder software, genes with the least stable value are considered as the most suitable and stable reference genes. In our analysis by NormFinder software, TBP gene with least stability value (SV=0.015) was selected as the reference for internal standardization in the different tissue under untreated and treated with herbicide. The expression stability of the reference genes Actin, GAPDH and Ef1α have decreased and the amount of their stability value have increased (Figure 3). Additionally, the software distinguished TBP and Actin genes (SV=0.012) as the best combination of two reference genes, combined by calculating the geometric mean (Table 3).

NormFinder ranked the candidate genes after their stability values (SV) based on the variations of their respective transformed-Ct values within and among groups (Table 3). The results indicated that TBP gene was stable under untreated and treated with herbicide and different tissues. While the Ef1α gene in herbicide treatment conditions and GAPDH gene in various tissues was recognized as the most unstable reference gene.

Delta-Ct method

Analysis by statistical delta-Ct method shows the difference in expression levels based on standard deviation, which lower SD represents a stable reference gene. Based on this method, TBP gene with lowest SD (3.7) and Ef1α gene with highest SD (14.39) was identified as the most stable and unstable reference gene for qPCR in wild oat, respectively (Figure 3).

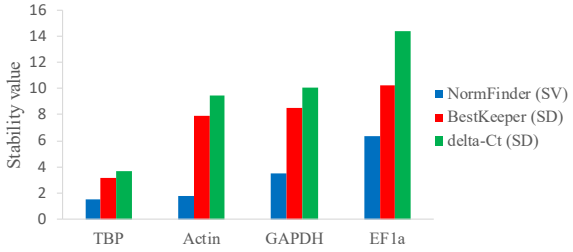

Figure 3. Ranking of candidate reference genes based on expression stability calculated in wild oat using NormFinder, BestKeeper and delta-Ct method.

Discussion

The aim of this study was to identify suitable reference genes for the normalization of gene expression data in wild oat plants in the different herbicide treatments and in the different tissues. In studies that use the qPCR technique, the target gene's mRNA should be normalized to the amount of mRNA used as internal reference gene for the purpose of data quantification. The software programs of NormFinder and BestKeeper and delta-Ct method are used in normalizing of mRNA amount of the internal reference gene(s) for qPCR technique. Using different statistical softwares, the best reference genes for normalization of expression data could be determined. We used NormFinder, which ranks candidate reference genes according to the variation of their expression within and among experimental modalities and is less sensitive to possible bias arising from co-regulation (26). Normalization is done to analysis the expression data using reference gene(s), which have stable expression levels in all cells and are not affected by experimental or environmental factors. That's while, many studies have already shown that the reference genes are regulated and vary under experimental conditions (27, 28). A number of studies have indicated

that optimum reference genes can even vary among different tissue samples (4, 18, 20, 29-31). In this regard, the expression patterns of reference genes in wild oat of non-target site resistance have been investigated under herbicide treatment in different tissues. The best reference gene selected in this study will help improve the quality of gene expression data among various wild oat samples.

The results of all softwares were similar (Figure 3), and indicated that TBP was the most stable gene in our experiment. Ranking of the candidate reference genes by NormFinder and BestKeeper showed TBP gene was the stable reference gene under untreated and treated with herbicide in different tissues. The TBP gene provides instructions for making a protein called the TATA box binding protein. This protein is active in cells and tissues, where it plays an essential role in regulating the activity of most genes. The phenomenon that reference genes have a stable expression in different cells, but stable reference genes may be different under different laboratory conditions. The results of ranking based on NormFinder and BestKeeper software showed that TBP gene was recognized as the most stable reference gene, while the Ef1α gene in herbicide treatment conditions and GAPDH gene in various tissues was recognized as the most unstable reference gene. Xu *et al.,* (32), in the study of reference genes in Japanese Foxtail (*Alopecurus japonicus*) response to herbicide, showed that 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.

Based on all statistical analyzes, TBP and Ef1α gens identified as the most stable and unstable reference genes for the subset of samples treated and untreated herbicide in different tissues in wild oat, respectively, that was similar to study by Wrzesińska *et al.,* (33). Ef1α gene has been reported in some grasses under biotic stress as unstable expression reference gene (20, 34). Petit *et al.,* (35), in the study on stability expression of 11 reference genes in grasses in response to herbicide treatment showed ubiquitin, beta-tubulin and GAPDH were identified as the best reference genes. Also they indicated that Ef1α and 18S were the least expression stably in genes study, while EF1 and 18S genes are among the most commonly used reference genes in plant studies, with a stable expression reported in grasses under different stresses in several studies (19, 36-38). Our research indicated that reference genes may be changed in the experimental conditions. However, our results and previous researches clearly confirmed the need for a thorough validation of the expression stability of candidate reference genes in the system considered prior to any gene expression study and analysis of qPCR data. We propose that TBP gene can be used as the internal control gene in future studies of qPCR for herbicide treatment in wild oat

Gene reference selection is one of the crucial steps in the qPCR analysis. Therefore, it is essential to study the stability of reference genes expression in various experiments before the qPCR analysis. The stability of four candidate reference genes of wild oat was assessed in two tissues under untreated and treated with herbicides stress. This study clearly indicated that the expression level of reference genes changes under different herbicides and tissues in wild oat. It was demonstrated that TPB is suitable reference gene for normalization in gene expression profiling studies for herbicide treatment in wild oat and potentially in other grass weed species. Our data showed that validation of candidate reference genes for each specific application must be conducted.

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