Expression Stability of Internal Reference Gene in Response to *Trichoderma polysporum* Infection in *Avena fatua* L.

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Abstract

In order to construct a RT-qPCR system suitable for response of Avena fatua L. to Trichoderma polysporum , and screen stable internal reference genes, GeNorm, NormFinder, BestKeeper and RefFinde were used to perform SYBR Green-based RT-qPCR analysis on 8 candidate internal reference genes (18S , 28S , TUA , UBC , ACT , GAPDH , TBP and EF-1) in A. fatua samples after inoculation of T. polysporum Strain HZ-31. The results showed that TBP , 18S and UBC were the most stable internal reference genes, TBP and TUA , TBP and GAPDH , 18S and TBP , UBC and 18S were the most suitable combination of the two internal reference genes, which could be used as the internal reference genes for functional gene expression analysis during the interaction between T. polysporum and A. fatua .

Introduction

The response of weeds to environmental stress is mediated by gene expression regulation. In modern agricultural system, the main stress faced by weeds invading farmland is the application of herbicides, which triggers the stress response pathway of weed plants (Délye 2013). To study the molecular mechanism behind this phenomenon and provide a basis for understanding the biological function of genes, it is necessary to analyze the gene expression patterns in plant stress response pathways. Gene expression patterns reflect the tendency of gene activity and are of great significance to the understanding of gene function and gene regulatory network (Gaines et al. 2014; Duhoux et al. 2015). Real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) is one of the most widely used techniques for gene expression analysis. It provides a means to compare the expression levels of target genes in different tissues and processes, enables researchers to perform quantitative analysis of genes, and also validates high-throughput gene expression profiles (Gachon et al. 2004). Using RT-qPCR to obtain reliable gene expression patterns in a given experimental system, it is necessary to eliminate or reduce the technical differences caused by the number and quality of templates, RNA extraction, cDNA synthesis efficiency and qPCR amplification itself (Kozera et al. 2013). Currently, standardization of RT-qPCR data gives priority to the use of internal reference genes as internal controls(Udvardi et al. 2008). Because of the physiological and biochemical processes necessary for cell survival, house-keeping genes (HKG) are normally expressed in a constant and unaffected manner and are a suitable candidate for RTRTP qPCR By internal reference genes (Kozera et al. 2013). By normalizing the target gene expression data relative to housekeeping genes (internal reference genes), whether the target gene expression level is up-regulated or down-regulated can be determined.

Although the expression level of internal reference genes is thought to be stable, it actually varies under different conditions (Pfaffl 2001) . Previous studies have shown that under a low temperature stress, the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression level in tomato (*Solanum lycopersicum* L.) plants increased by 30 times, so this gene is not used as an internal reference gene for tomato gene expression analysis involving temperature changes (Løvdal et al. 2009). The use of an intrinsic gene without prior verification of its expression stability may lead to erroneous experimental results which may lead to erroneous conclusions about the biological effects (Dheda et al. 2005). Therefore, the use of statistical methods to identify the most suitable candidate genes in each particular laboratory or biological environment has become a prerequisite for qPCR analysis (Kozera et al. 2013).

Because most weeds are species without related genomic resources, only a few internal reference genes are used in weeds by scholars, and most of them are in the study of response mechanism of weeds to chemical herbicides (Duhoux et al. 2013; Petit et al. 2012), and the infection of *Trichoderma polysporum* belongs to biological stress, and the response mechanism of *Avena fatua* L. is different, it is necessary to revalidate the internal reference genes to analyze the gene expression of *T. polysporum* which is specific to *A. fatua* Therefore, in this study, RT-qPCR based on SYBR reagents was used to identify internal reference genes in *A. fatua* in response to the minimal expression changes during the infection of *T. polysporum*. Through preliminary screening of 40 internal reference genes commonly used in plants, 8 internal reference genes were finally selected as candidate internal reference genes for evaluation, including *18S* ribosomal RNA (*18S* rRNA), *28S* ribosomal RNA (*28S* rRNA), α-tubulin (*TUA*), ubiquitin conjugating enzyme (*UBC*) and *ACT*in (*ACT*in), glyceraldehyde-3-

phosphate dehydrogenase (*GAPDH*), TATA binding protein (*TBP*) and translation elongation for *ACT* (EF-1) (Wang et al. 2014). Four different methods were used to evaluate the expression stability of these genes in order to select the most suitable internal reference genes.

Materials And Methods

Strain for Test

T. polysporum isolated from naturally sensitive plants was provided and preserved by the Comprehensive Control Laboratory of Plant Protection Institute of Qinghai Academy of Agricultural and Forestry Sciences.

Materials for Test

Two groups of *A fatua* seedling samples were treated with *T. polysporum* fermentation filtrate and the control group was treated with water.

Reagents for Test (kit)

Liquid nitrogen, RNA extraction Kit (RNAsimple Total RNA Kit, TIANGEN), genomic DNA contamination removal and the first strand cDNA synthesis kit (PrimeScript^{TM} RT reagent Kit with gDNA Eraser, TaKaRa), RT-qPCR kit (SYBR$^{®}$ Premix Dimer Eraser^{TM}, TaKaRa).

A. fatua Sample Preparation

After the seeds were sterilized and sprouted, the seeds were sown in plastic flowerpots (20 cm in diameter and 14 cm in height), 50 seeds per pot, and a total of 18 pots, and they are cultivated in a greenhouse after sufficient watering with routine management. The day or night temperature of the greenhouse is 25 ± 1 ℃ or 20 ± 1℃, the relative humidity is from 65% to 75%, and the light intensity is from 600 to 800 μmol m^{-2} s^{-1}. Seedlings were thinned once they have two sets of leaves, then they were divided into two groups randomly and 30 consistent and strong seedlings are retained in each pot. One group was treated with *T. polysporum* fermentation filtrate with 10^8 per mL of the spore concentration and each pot is evenly sprayed 10 mL, and the other group was sprayed with equal amount of water. The above-ground samples were collected separately before the treatment, 3 days after the treatment and 6 days after the treatment, quickly frozen in liquid nitrogen and then stored in a refrigerator at -80℃ for later use. Three biological replicates were performed for each treatment type and treatment time.

Total RNA Extraction and First Strand cDNA Synthesis

According to the Kit instructions, Total RNA was isolated from *A. fatua* samples using RNAsimple Total RNA Kit. The mass and concentration of RNA were determined by ultra-micro spectrophotometer, and samples with A260/A280 ratio between 1.8 and 2.2, and A260/A230 ratio >2.0 are kept. Using PrimeScript^{TM} RT reagent Kit with gDNA Eraser kit, genomic DNA contamination removal and first strand cDNA synthesis were carried out according to the operating instructions.

Candidate Internal Reference Genes and Primers

In order to screen the internal reference genes suitable for the quantitative PCR study of *A. fatua* gene expression, 40 internal reference genes commonly used in plants were selected on ICG (http://icg.big.ac.cn/index.php/Main_Page) (Sang et al. 2017). Based on the specificity of amplification results and the consistency of amplification conditelons, 8 commonly used housekeeping genes were finally identified as candidate genes, including *18S* ribosomal RNA (*18S*), *28S* ribosomal RNA (*28S*), Tubulin alpha (*TUA*), ubiquitin-conjugating enzyme (*UBC*), *ACT*in (*ACT*), glyceraldehyde 3-phosphate

dehydrogenase (*GAPDH*), TATA-binding protein (*TBP*) and elongation factor (EF-1a). The primer sequences are shown in Table 1.

Table 1 Candidate Internal Reference Genes and Primers Sequence

Gene name	Primer sequence (5-3, forward/reverse)	Product length	Amplification efficiency
18S	GTGACGGGTGACGGAGAATT/ GAC*ACT*AATGCGCCCGGTAT	151	98%
28S	CCTGATCTTCTGTGAAGGGTTCGA/ GGTTCGATTAGTCTTTCGCCCCTA	172	96%
TUA	GTGCCTACCGTCAGCTTTT/ CGATCTCCTTTCCAACAGTGT	102	94%
UBC	ACAGTAACGGGAGCATATGTC/ GGTCCGTTAGCAGAGAACAG	102	99%
ACT	GTAACATTGTGCTCAGTGGTG/ T*ACT*TCCTCTCGGGTGGTG	127	93%
GAPDH	TTGATCTCACCGTCAGAATCG/ TGCTGTCGCCAATGAAGTC	118	109%
TBP	ATGGTGCTTTCTCAAGTTATG/ CGAAGGCAGTGTATGTCTC	112	105%
EF-1a	CAAGAATGTTGCCGTGAAGG/ GCCGTTGCCAATCTGACC	133	99%

Real-time Quantitative PCR Analysis

The RT-qPCR analysis was performed on Roche LightCycler96 real-time fluorescent quantitative PCR system using SYBR® Premix Dimer Eraser™. The reaction system was 20μL, and the reaction mixture consisted of 2μL cDNA template, 10μL 2 × SYBR® Premix DimerEraser, and up and down primer 0.8μ L (10μM) and 6.4μL ddH_2O, respectively. The amplification conditions were as follows: denaturation at 95°C for 5 min; denaturation at 95°C for 10 s, extension at 60°C for 30 s, 40 cycles. The melting curve is generated by raising the amplification temperature from 60°C to 95°C, with each cycle temperature rising by 0.5°C. Setting without template control, RT-qPCR analysis of each sample contains 3 technical replicates.

Analysis of Candidate Gene Expression Stability

The stability of 8 candidate internal reference genes was analyzed by using four programs: GeNorm (Vandesompele et al. 2002; Yang et al. 2015), NormFinder (Yang et al. 2015; Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004; Migocka et al. 2011)and RefFinder (Silver et al. 2006; Xie et al. 2012). The primer specificity was verified by the presence of a single peak in RT-qPCR melting curve determination. The RT-qPCR amplification efficiency of each intrinsic gene was measured by LinReg PCR software (Ruijter et al. 2009). Theoretically the optimal value is 100%, indicating that the template is copied exponentially, and the acceptable range is usually from 90% to 110% (Sgamma et al. 2016).

Results

Primers Performance Analysis

The results obtained from quantitative analysis showed that the primer dimer and additional bands were not observed and a single-peak pattern was formed (Figure 1). The single-peak melting curve confirmed that there was no primer dimer and non-specific amplification in the gene studied. The efficiency of PCR was from 93% (*ACT*) to 109% (*GAPDH*) (Table 1).

Expression Level Variation of Candidate Internal Reference Gene

Cq value characterizes mRNA transcription level. The Cq value from RT-qPCR analysis can be used to detect the expression level of 8 candidate internal reference genes. Table 4-2 lists the expression levels of each candidate internal reference gene. Under different treatments and time, the Cq level of all candidate internal reference genes ranged from 12.8 to 28.1. *28S* has the highest expression level, with an average Cq level of 12.8, while *TUA* has the lowest expression level with an average Cq level of 25.8 (Table 2). The coefficient of variation (CV) shows the degree of variation of measured values in each treatment (lower values indicate lower variability). Under all treatments and treatment time, *18S* variability was the lowest with the CV value of 2.6%, and variability was the highest with the maximum CV value of 11.2%.

Table 2 Expression Levels of 8 Candidate Internal Reference Genes

Treatment	Processing Time	*18S*	*28S*	*TUA*	*UBC*	*ACT*	*GAPDH*	*TBP*	EF-1a
CK	0 d	16.1	13.3	27.4	22.0	25.1	21.2	19.3	26.8
	3 d	16.2	12.7	27.0	22.2	26.1	21.0	18.5	25.6
	6 d	16.4	13.1	28.1	22.3	26.2	20.5	19.0	25.7
HZ-31	0 d	16.2	12.7	27.0	22.0	23.8	20.1	18.6	23.2
	3 d	16.1	12.8	23.8	23.2	24.5	20.7	18.9	22.8
	6 d	16.1	12.9	21.2	22.8	24.5	20.4	19.1	24.2
Average		16.2	12.9	25.8	22.4	25.0	20.6	18.9	24.7
Standard deviation (SD)		0.4	0.5	2.8	1.4	1.6	1.3	0.7	2.8
Coefficient of (Variation CV/%)		2.6	3.8	10.9	6.1	6.4	6.1	3.6	11.2

GeNorm Analysis

The program GeNorm uses the M value to evaluate the stability of 8 candidate internal reference genes. The M value is the average variation of a gene relative to all other genes. In the program GeNorm, the threshold to determine gene stability is set to M < 1.5, and the lower the M value, the higher the candidate gene stability (Vandesompele et al. 2002). Figure 2 shows the average expression stability M value of candidate internal reference genes based on geNorm analysis. It can be seen that the M value of most candidate genes is less than 1.5, which indicates that the expression stability of 8 candidate internal reference genes is good. The *28S* and *TBP* with the same M value were the most stable internal reference genes during the normal growth of *A. fatua*, while *GAPDH* and *TBP* were the most stable internal reference genes in the above-ground tissues of *A. fatua* of Day 3 treated with *T. polysporum* fermentation filtrate. *18S* and *28S* were the most stable internal reference genes in the above-ground tissues of *A. fatua* of Day 6 treated with *T. polysporum* fermentation filtrate and were also the most stable internal reference genes including all the treated samples (Total). In most samples, *EF-1*a and *TUA* were identified as the most unstable internal reference genes.

Total refers to the combination of all processed samples, and the same below.

In order to determine the optimal number of internal reference genes, NF_n and NF_{n+1} were used in the program GeNorm to calculate V_n/V_{n+1} values of pairs of different candidate genes, and to determine whether increasing the number of internal reference genes could improve the stability of standardized factors. Usually, 0.15(V_n value) is used as the termination value (Vandesompele et al. 2002). Figure 3 shows the best internal parameter number analysis for different treatments based on GeNorm. The $V_{2/3}$ of Day 0, Day 3, Day 6 and all processed samples (Total) treated with *T. polysporum* fermentation filtrate were 0.131, 0.116, 0.128 and 0.090, respectively, which were lower than 0.15 (Figure 3), showing that the two internal reference genes were sufficient for the accurate normalization of these samples. However, the $V_{3/4}$ of Day 0, Day 3 and Day 6 and all processed samples (Total) treated with *T. polysporum* fermentation filtrate were different, and the $V_{3/4}$ of Day 0

and all treated samples (Total) were 0.176 and 0.189, respectively, which were more than 1.5; the $V_{3/4}$ of Day 3 and Day 6 were 0.090 and 0.103, respectively, which were less than 1.5, and the $V_{4/5}$ were more than 1.5 (0.284 and 0.177).

NormFinder Analysis

Using the program NormFinder, the stability values of 8 candidate intrinsic parameters were calculated. The lower the values, the higher the stability (Table 3). It can be seen that the three most Total internal reference genes were *UBC* (0.100), *GAPDH* (0.100) and *ACT* (0.338) in all treated samples. *TUA* and *UBC* were the most stable genes expressed in the samples in Day 0, while *EF-1*a was the least stable internal reference gene expressed in the samples. *UBC* and *GAPDH* were the most stable internal reference genes in the samples treated for 3 days, and *EF-1*a was the most stable internal reference gene in the samples treated for 3 days, consistent with the GeNorm data. *GAPDH* and *ACT* were the most stable genes expressed in the samples treated for 6 days.

However, in most of the samples, the stability level of the candidate intron gene generated by NormFinder was different from that of GeNorm. For example, in the GeNorm analysis, *18S* and *28S* were identified as the most stable intrinsic genes in all processed samples (Total), while in the NormFinder analysis, they ranked sixth and fifth, respectively.

Table 3 Stability of Candidate Internal Reference Gene Analysis Based on NormFinder

Ranking	1	2	3	4	5	6	7	8
0 d	*TUA* (0.192)	*UBC* (0.327)	*TBP* (0.335)	*28S* (0.415)	*ACT* (0.455)	*GAPDH* (0.461)	*18S* (0.664)	EF-1a (1.103)
3 d	*UBC* (0.402)	*GAPDH* (0.540)	*TBP* (0.546)	*18S* (0.631)	*28S* (0.744)	*TUA* (0.973)	*ACT* (0.986)	EF-1a (1.188)
6 d	*GAPDH* (0.180)	*ACT* (0.207)	*TBP* (0.508)	*UBC* (0.529)	*18S* (0.789)	*28S* (0.794)	EF-1a (1.384)	*TUA* (1.886)
Total	*UBC* (0.100)	*GAPDH* (0.100)	*ACT* (0.338)	*TBP* (0.514)	*28S* (0.713)	*18S* (0.738)	EF-1a (1.090)	*TUA* (1.131)

Total refers to the combination of all processed samples with the stability value in parentheses, the same below.

BestKeeper Analysis

Based on the Cq value, the expression stability of 8 candidate internal reference genes was analyzed by the program BestKeeper, and the variation coefficient (CV) and standard deviation (SD) of all candidate reference genes were calculated. The data of SD < 1 were considered as acceptable variation range, and the higher CV and SD value indicated the lower gene expression stability (Migocka and Papierniak, 2010). Table 4 shows the results of the BestKeeper analysis. Under the conditions of this experiment, *18S* was the most stable gene of Day 0, Day 6 and all the processed samples (Total) treated with *T. polysporum* fermentation filtrate, and *28S* was the most stable gene expressed in the treated samples of Day 3 with its standard deviation of less than 1 (SD < 1). The expression stability of *GAPDH* was ranked second in the samples of Day 3, but lower in Day 0, Day 6 and all processed samples (Total). *EF-1*a and *TUA* were rated as the most unstable gene in most of the samples, except that the standard deviation of *TUA* was 0.98 in Day 0, and the standard deviation of both was greater than 1 (SD > 1). Similarly, for most of the samples, the expression stability of candidate internal reference genes generated by BestKeeper was inconsistent with the results of GeNorm and NormFinder.

Table 4 Stability of Candidate Internal Reference Genes Analysis Based on BestKeeper (CV±SD)

Ranking	1	2	3	4	5	6	7	8
0 d	*18S* 1.14±0.18	*TBP* 2.64±0.50	*TUA* 3.62±0.98	*ACT* 3.67±0.90	*28S* 3.70±0.48	*UBC* 4.85±1.07	*GAPDH* 6.95±1.43	EF-1a 7.32±1.83
3 d	*28S* 1.96±0.25	*GAPDH* 2.40±0.50	*TBP* 2.61±0.49	*18S* 2.69±0.43	*ACT* 5.40±1.37	*UBC* 6.60±1.50	*TUA* 7.96±2.02	EF-1a 9.18±2.22
6 d	*18S* 2.26±0.37	*UBC* 2.81±0.63	*28S* 2.95±0.38	*TBP* 3.50±0.67	*ACT* 4.91±1.24	*GAPDH* 5.72±1.17	EF-1a 11.01±2.75	*TUA* 14.13±3.48
Total	*18S* 2.08±0.34	*28S* 33.03±0.39	*TBP* 3.11±0.59	*ACT* 5.03±1.26	*UBC* 5.04±1.13	*GAPDH* 5.11±1.05	*TUA* 9.13±2.35	EF-1a 9.32±2.31

RefFinder Analysis

The comprehensive ranking of candidate internal reference genes is determined by the program RefFinder (Table 5). The program RefFinder integrates four analysis programs, including GeNorm, NormFinder, BestKeeper, and the delta Ct evaluation method. The results showed that *TBP* was the suitable internal reference gene in the gene expression analysis of *A. fatua* samples of Day 0 and Day 3 treated with *T. polysporum* fermentation filtrate, *18S* was the suitable internal reference gene of Day 6, and *UBC* was the most suitable internal reference gene of all processed samples (Total). In all treatments, *EF-1*a and *TUA* were the most unstable candidate internal reference genes.

The use of multiple internal reference can effectively reduce the test error. Under the conditions of this experiment, the combinations of two suitable internal reference genes samples of Day 0, 3, 6, and all processed samples (Total) treated with *T. polysporum* fermentation filtrate are *TBP* and *TUA*, *TBP* and *GAPDH*, *18S* and *TBP* , *UBC* and *18S*.

Table 5 Stability of Candidate Internal Reference Genes Analysis Based on RefFinder

Ranking	1	2	3	4	5	6	7	8
0 d	*TBP* (2.06)	*TUA* (2.11)	*28S* (2.21)	*18S* (3.48)	*UBC* (3.94)	*ACT* (5.18)	*GAPDH* (6.19)	EF-1a (8.00)
3 d	*TBP* (1.73)	*GAPDH* (2.00)	*28S* (2.78)	*18S* (3.13)	*UBC* (3.50)	*ACT* (6.19)	*TUA* (6.48)	EF-1a (8.00)
6 d	*18S* (2.24)	*TBP* (2.45)	*28S* (2.91)	*UBC* (3.13)	*GAPDH* (3.31)	*ACT* (3.66)	EF-1a (7.00)	*TUA* (8.00)
Total	*UBC* (2.24)	*18S* (2.45)	*28S* (2.66)	*GAPDH* (2.83)	*TBP* (3.22)	*ACT* (4.56)	EF-1a (7.00)	*TUA* (8.00)

In this study, we performed the first comprehensive evaluation of stable internal reference genes required for RT-qPCR analysis of target gene expression in the above-ground tissues of *A. fatua* treated with *T. polysporum* strain HZ-31 fermentation filtrate. Through the evaluation of 4 softwares, *TBP*, *18S* and *UBC* were the most stable internal reference genes expressed in *A. fatua* infected by *Trichoderma* spp., which have good statistical reliability and can be used as the internal reference genes in molecular mechanism study of *A. fatua* in response to *Trichoderma* spp. infection. *EF-1*a and *TUA* are the most unstable internal reference genes.

TBP, 8S and *UBC* were the most suitable internal reference genes for *A. fatua* samples of Day 0, Day 3 and Day 6 treated with fermentation filtrate. *TBP* and *TUA*, *TBP* and *GAPDH*, *18S* and *TBP*, *UBC* and *18S* were the most suitable gene combinations *TBP*BP, *18S* d *UBC* were the most suitable internal reference genes for *A. fatua* treated with HZ-31 fermentation filtrate for 0, 3 and 6 days. *TBP* and *TUA*, *TBP* and *GAPDH*, *18S* and *TBP*, *UBC* and *18S* were the most suitable gene combinations. In addition, the use of three reference genes is enough to reliably standardize the target genes in *A.*

fatua. No absolute quantitative analysis of reference genes is recommended because the lack of normalization can lead to inaccurate or incorrect conclusions.

Discussion

A. fatua is one of the most common and economically harmful weed species in the world (Beckie et al.2017; Wrzesińska et al.2016), which is highly competitive, resistant to a variety of herbicides, mimics crops (similar to phenology) and has different germination strategies, and is extremely difficult to eliminate (Wrzesińska et al.2016). *Trichoderma* spp. is an important biocontrol strain. The strain HZ-31 *T. polysporum* screened in this study has a good control effect on *A. fatua*(Zhu et al.2020). In order to study the biological control mechanism of *Trichoderma* spp., it is necessary to analyze the response mechanism of weed to *Trichoderma* spp. and to study the gene expression pattern in response pathway. RT-qPCR is one of the widely used techniques for quantitative analysis of gene expression. To analyze gene expression patterns by RT-qPCR, the original data must be standardized to mitigate the effects of technology and manipulation, which is usually achieved by normalization of internal reference genes (Kozera et al. 2013; Udvardi et al. 2008; Despiegelaere et al. 2015). The expression of good internal reference genes should not be affected by experimental conditions, and the expression level should be stable in all cells. However, many studies have shown that some of the internal reference genes are regulated and changed under experimental conditions, and the optimal internal reference genes are different in different tissue samples (Bhatia et al. 1994; Zhang et al. 1992; Jarošová et al. 2010). Therefore, in this study, RT-qPCR analysis based on SYBR Green was performed on 8 candidate internal reference genes (*18S*, *28S*, *TUA*, *UBC*, *ACT*, *GAPDH*, *TBP* and *EF-1*a).

GeNorm, NormFinder, and BestKeeper are three programs based on statistical analysis that researchers use to analyze the expression stability of internal reference genes (Silver et al. 2006; Xie et al. 2012). NormFinder operates like the program GeNorm, but the latter can select the right combination of reference genes and the best number of reference genes. Compared with GeNorm and NormFinder, the program BestKeeper can analyze the stability of reference genes and target genes. In this study, the analysis results of GeNorm and NormFinder were similar, while those of BestKeeper were quite different. Previous studies have also reported the differences between BestKeeper and other methods (Rapacz et al. 2012). In several studies, the differences among different methods are solved by ranking geometric averages based on multiple evaluation methods, including Web-based tool RefFinder (Wrzesińska et al. 2016; Wang et al. 2016; Taylor et al. 2016) and cross entropy algorithm provided by weighted aggregate R package RankAggreg (Mallona et al. 2010). Therefore, this study used four programs (GeNorm, NormFinder, BestKeeper, and RefFinder) to select the internal reference genes of *Avena fatua* L, and similar methods have been used in many species in previous studies (Marianne et al. 2015; Chen et al. 2015).

TBP, *18S* and *UBC* were the most stable internal reference genes expressed in *A. fatua* infected by *Trichoderma* spp., and *EF-1*a and *TUA* were the most unstable internal reference genes, which was similar to previous studies. *TBP*, a TATA box-binding protein, is highly *ACT*ive in cells and tissues and plays an important role in regulating the activity of most genes. *UBC* is the only stable expression of internal reference gene (Silveira et al. 2009)in Arabidopsis and tomato seeds. It is reported that *UBC* has high expression stability and is the only stable expression of internal reference gene in seeds of Arabidopsis and tomato. In herbicide-treated *Avena fatua* L, the *GAPDH* gene is also considered one of the most stable genes (Wrzesińska et al. 2016). In contrast, the relatively unstable expression of *GAPDH* in this study may reflect divergent reports on the stability of *GAPDH* gene expression. For example, *GAPDH* is recommended as a suitable reference gene for the seed development of tung tree (Han et al. 2012), but the results of Euphorbia show that the transcriptional levels of *GAPDH* 1 and *GAPDH* 2 are very unstable in buds, seeds and various organs (Chao et al. 2012). This suggests that the contrast between different species and even within the same species may not be due to the differential expression of different treatments, but may also be due to the heterogeneity of different primers, which may amplify the members of different species and thus present a differential expression profile.

Hongle et al. (Hongle et al. 2017) found that EF1 and UBQ in roots, EF1, TUB, CAP and *18S* in stems and *GAPDH* and *18S* in leaves could be used as qPCR-normalized internal reference genes after studying the internal reference stability of herbicide-

responsive genes in Alopecurus japonicus. *EF-1*a gene was reported as an unstable expression reference gene in some weed plants under biological stress (Jarošová et al. 2010; Faccioli et al. 2007). In the study of the stable expression of 11 reference genes in herbicide treatment, Petit et al. (2012) pointed out that *EF-1*a and *18S* were the stable genes with the least expression, so the stability of reference genes could be changed due to different species. In this study, the reference gene was changed under the experimental conditions, *GAPDH* had good stability in Day 3 after *A. fatua* treated by *T. polysporum*, but the stability was lower in other time, so it was necessary to verify the candidate reference gene for each specific application, and to fully verify the expression stability of candidate internal reference genes.

In terms of the number of optimal reference genes, it's found the $V_{3/4}$ of Day 3 and Day 6 were less than 1.5 and smaller than $V_{2/3}$ by GeNorm analysis, which suggested that three genes (*GAPDH*, *TBP* and *28S* or *18S*, *28S* and *TBP*) might be needed to effectively standardize the samples of Day 3 and Day 6. However, 1.5 should not be used as a strict criterion for terminating values. Studies have found that V_n/V_{n+1} has a higher terminating value (Yang et al. 2015; Chen et al. 2015;Silveira et al. 2009). Robledo et al. (2014) criticised the behavior of choosing the best endoparametric genes based on non-biological ranking, and suggested that the reference genes provided by NormFinder shuold be supported by BestKeeper's descriptive statistics (SD, CV, and r) if the four approaches were different. Kozera and Rapacz (Kozera et al. 2013) recommend using at least one pair of genes responsible for greater functional differences because they were less likely to be expressed simultaneously. Others recommend using at least three reference genes to get the best results (Bustin 2010; Derveaux et al. 2010).

Conclusions

TBP, *18S* and *UBC* were the most stable internal reference genes for *A. fatua* infected *T. polysporum*. *TBP* and *TUA*, *TBP* and *GAPDH*, *18S* and *TBP*, *UBC* and *18S* were the most suitable combination of two internal reference genes, which laid a foundation for the expression analysis of functional genes and the study of pathogenic mechanism, and it is also helpful to further study the gene interaction between *T. polysporum* and *A. fatua*.

Declarations

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Availability of data and materials All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interests The authors declare no conficts of interest.

Ethical statement This manuscript is not under consideration for publication elsewhere, and all authors approve submission and declare that no competing interests exist.

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Figures

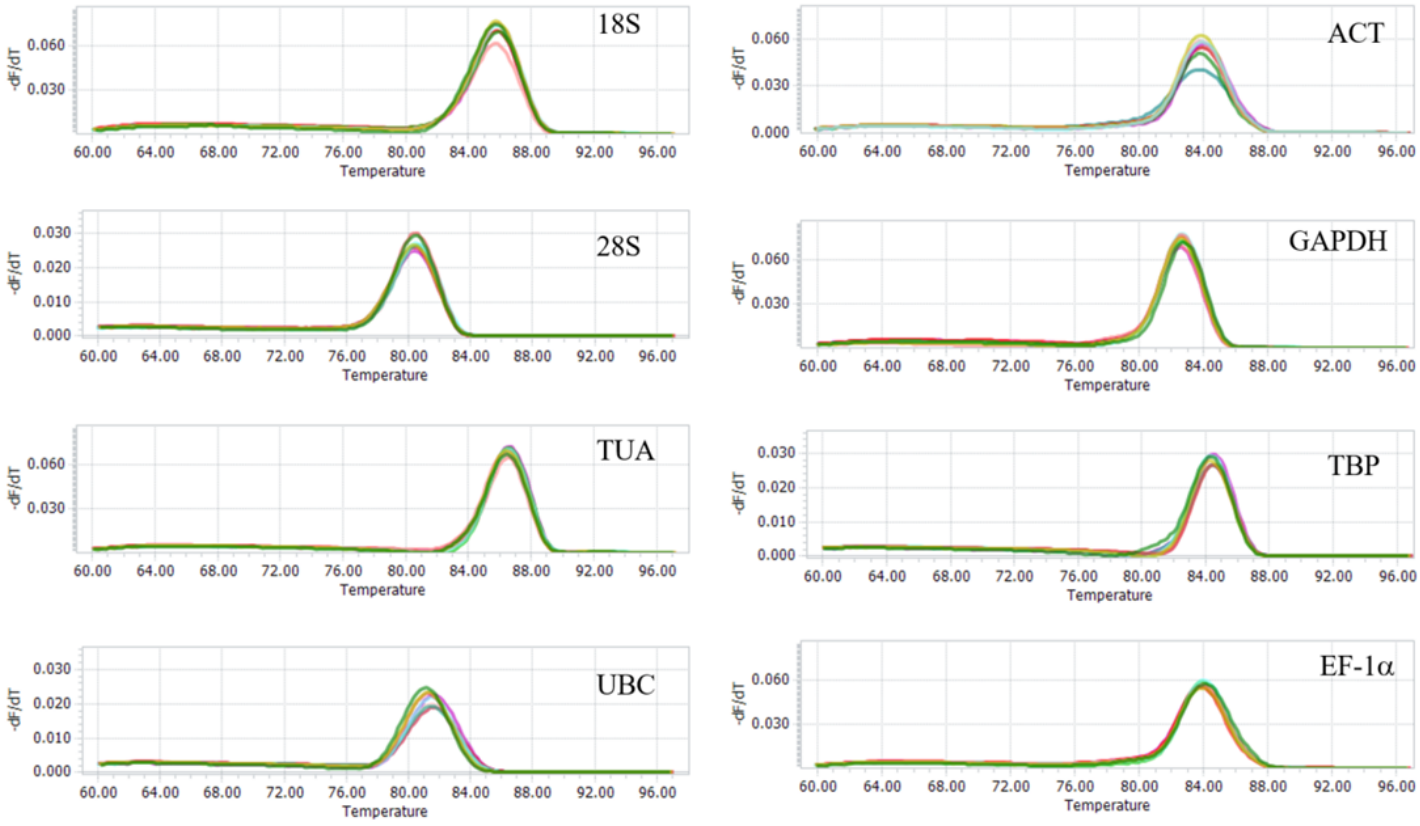

Figure 1

Dissolution Curve of Candidate Internal Reference Primer RT-qPCR

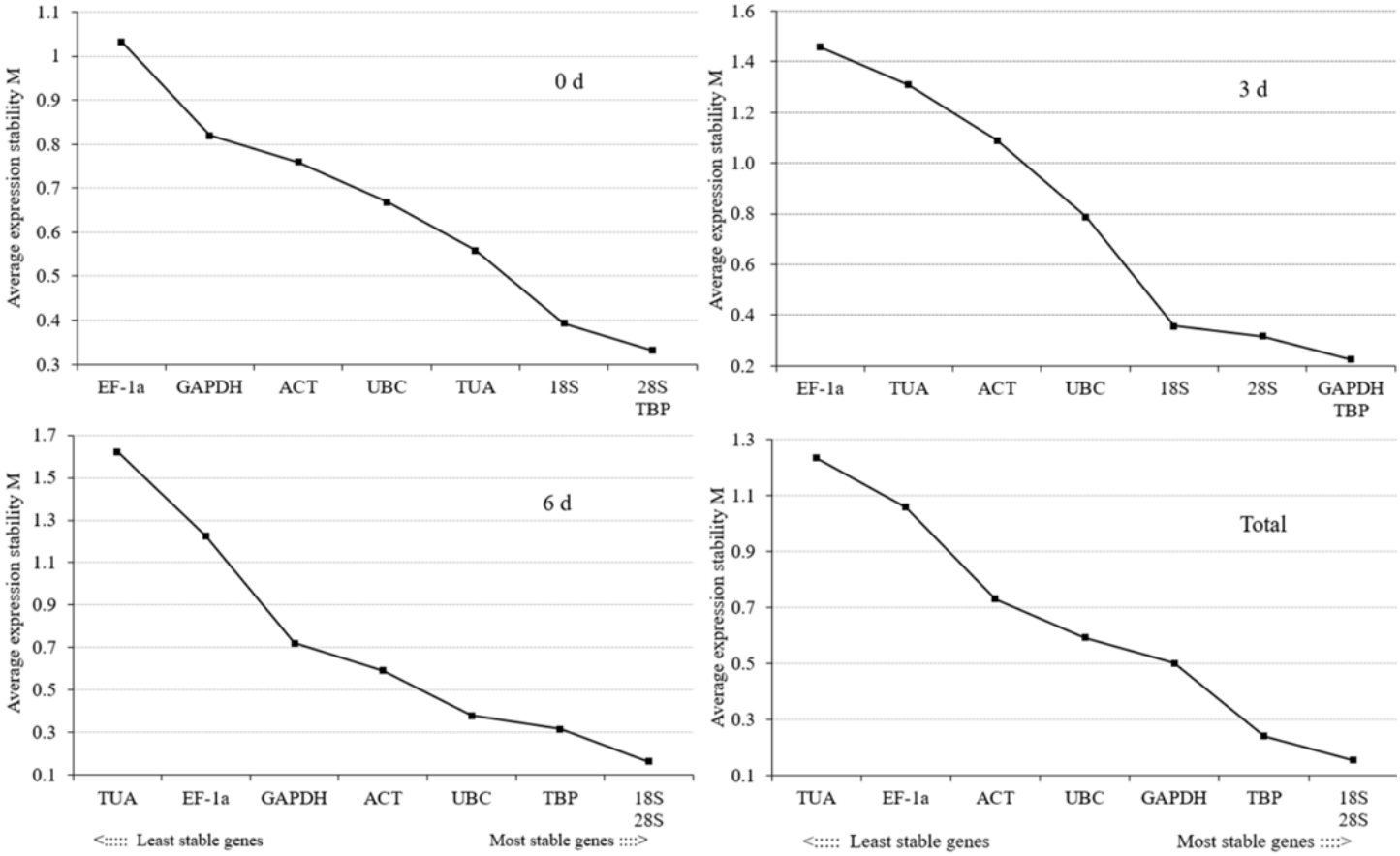

Figure 2

Average Expression Stability of Candidate Internal Reference Genes Based on geNorm Analysis

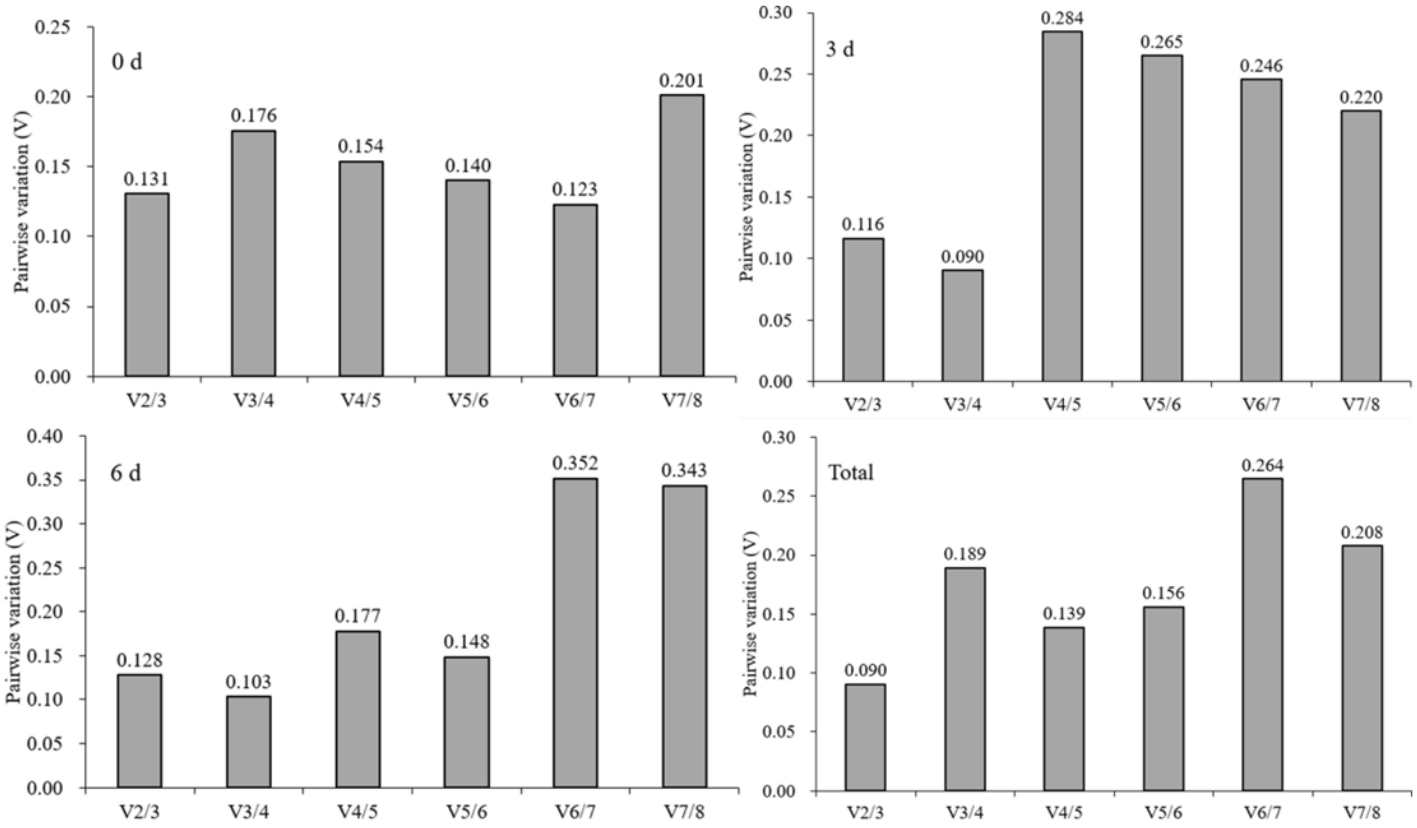

Figure 3

Optimum Internal Reference Gene Quantity Analysis under Different Treatment Based on geNorm