https://doi.org/10.56093/ijas.v90i1.98678

Indian Journal of Agricultural Sciences **90** (1): 189–94, January 2020/Article

Evaluation of stable reference genes in white mustard (*Sinapis alba*) for qRT-PCR analysis under various stress conditions

SHIKHA DIXIT[1], VINOD KUMAR JANGID[2] and ANITA GROVER[3]*

ICAR-National Institute for Plant Biotechnology, Pusa, New Delhi 110 012, India

Received: 25 June 2019; Accepted: 05 July 2019

ABSTRACT

White mustard (*Sinapis alba* L.) is a member of Brassicaceae family and is a source of various biotics stress resistance genes. *S. alba* is closely related to the cultivated *Brassica juncea* which exhibit susceptibility towards various pathogens. Quantitative real-time PCR (qRT-PCR) is an efficient method to estimate the gene expression levels but the efficiency of its outcome is largely influenced by the stability of the reference gene. Many studies have reported considerable variation in the expression of reference genes in different tissues and treatments therefore, screening for accurate reference genes is important for functional analysis of the target gene. This study was conducted with the aim of identifying suitable reference genes for efficient quantitative gene expression analysis in *S. alba*. This experiment was conducted in National Phytotron Facility, IARI, Pusa campus in the month of November-December 2017. In this study, stability of seven candidate reference genes were identified across diverse samples of *S. alba* representing- hormone treated, wounded and *A. brassicae* inoculated samples. Results revealed that TIPS41 and PP2A the overall best performing reference genes in *S. alba*. However, best-ranked reference gene should be selected according to the specific sample subset.

Key words: geNorm, Normfinder, Reference genes, qRT-PCR, *Sinapis alba*

Gene expression analysis provides important understanding of the complex metabolic pathways and signaling networks responsible for plant's physiological response to various abiotic and biotic stresses. qRT-PCR is a sensitive and accurate method to evaluate the expression of target gene in different tissues and treatments. However, factors such as RNA quality and PCR efficiency greatly influence the overall accuracy of the experiment (Gachon *et al*. 2004). To obtain an efficient gene expression analysis selecting stable expressing reference genes under different experimental conditions is crucial (Kundu *et al*. 2013). Many previous reports have reported differential expression of housekeeping genes under different tissue type and experimental conditions (Duan *et al*. 2017). Majorly, traditional housekeeping genes like actin 7 (ACT7), Elongation factor 1- alpha (EF1A), polyubiquitin (UBQ) and tubulin alpha (TUA) are used as reference genes (Gutierrez *et al*. 2008). However, in recent years many new reference genes have been identified as suitable reference genes in species like *A. thaliana* (Czechowski *et al.* 2005), *B. juncea* (Chandna *et al*. 2012), *B. napus* (Yang *et al*. 2014), tobacco (Schmidt & Delaney, 2010) and rice (Jain *et al.* 2006).

Statistical algorithms like geNorm (Vandesompele *et al*. 2002) and Normfinder (Andersen *et al*. 2004) have been developed in the past years to analyze the expression pattern of housekeeping gene and identify the stable expressing gene. These software compares the pairwise expression of each gene and identify parameters like stability value, inter and intra group variability and minimum number of reference genes needed for accurate expression analysis. White mustard (*S. alba*) is oilseed crop which is widely used as spice, food and fodder. *S. alba* exhibit moderate resistance against the *A. brassicae* and it is well known fact that phytohormones like SA, JA and ABA and their cross-talk play a central role in determining the plant's response to a particular pathogen (Pieterse *et al*. 2012). In the current study, we compared the expression of 7 candidate reference genes across 48 diverse samples subjected to various treatments. Statistical algorithm geNorm and NormFinder were utilized for the analysis in order to identify suitable reference gene for expression analysis in *S. alba*.

MATERIALS AND METHODS

Plant material and stress treatment: S. alba plants were grown in a controlled environment in growth chamber of national phytotron facility, IARI, New Delhi in November-December 2017. The growth chambers were programmed

---

[1,2]Ph D Student (dixitshikha0@gmail.com, vinod_shikher@yahoo.co.in), [3]Principal Scientist (anitagrover@hotmail.com), ICAR-National Institute for Plant Biotechnology, Pusa, New Delhi.

for 16h/8h of light/dark cycle at a temperature of 24°C/20°C for day/night. 35 days old plants were subjected to various treatments and samples were collected at different time interval (0, 3, 6, 12, 24, 48, 72, 96 h). For hormone-treatment, plants were sprayed with SA (1mM), MeJA (100µM) and ABA (100µM) solution. For wounding-treatment, leaf surface was scratched lightly with the help of blunt forceps without breaking the leaf. For *Alternaria brassicae* treatment, fungus was cultured in half strength potato dextrose agar (PDA) and spore suspension (5×10^6 spores/ml) prepared with 15 days old culture plate was used to inoculate the plants. Control plants were maintained simultaneously for each treatment.

Total RNA isolation and cDNA synthesis: The TRIzol^{TM} reagent (Invitrogen) was used for total RNA extraction from frozen leaf tissues. Purity and quantity of RNA were determined using NanoDrop^{TM} 2000 spectrophotometer (Thermo Scientific). Samples with $A_{260}/A_{280} > 1.8$ and $A_{260}/A_{230} > 2$ were used further for cDNA synthesis. First strand cDNA synthesis kit (Thermo Scientific) was used to synthesize the first strand cDNA from 1µg of RNA for 20 µl of reaction with oligo dT primers. cDNA was diluted to 1:10 (cDNA: nuclease free water) for further qPCR reactions.

Candidate reference genes selection and primer designing: Total 7 candidate reference genes were selected for the experiment- ACT7 (actin 7), EF1A (elongation factor 1 alpha), UBQ9 (ubiquitin 9) and TUB1A (tubulin alpha), TIPS41 (tonoplastic intrinsic proteins 41), CAC (Clathrin adopter complex) and PP2A (protein phosphatase 2A). To design the primers of genes, Arabidopsis CDS sequence was gathered from GenBank and used as a query sequence in BLASTn to obtain the homologous sequences of various Brassicaceae wild type from *B. rapa* genome portal (http://brassicadb.org/brad). Sequences of each gene were aligned using CLASTALW tool and primers were designed from the consensus region of the aligned sequences. For all the genes, primers were designed using online tool Primer 3 (Untergasser *et al.* 2012) with following parameters: primer length 20-25 bp, amplicon size 100-200 bp, GC content 60-65%, melting temperature 60-65°C, absence of hairpin structure, homodimer and heterodimer. To check the specificity of the designed primer, melt curve analysis was done. Amplification efficiency (E) and correlation coefficient (R^2) were calculated based on the slope of standard curve prepared from a series of dilutions as per following equation: $E (\%) = (10^{-1/slope}-1)\times100$. Primer details and amplification efficiency is shown in Table 1.

Quantitative Real time PCR (qRT -PCR): qRT- PCR was done using SYBR Green technology in 96 well optical plate with LightCycler□ 480 real time PCR machine (Roche) adopting following thermocycle conditions: 95°C for 5min, 40 cycles of 95°C for 40 sec, 60°C for 60 sec and 72°C for 40 sec. Melt curve analysis was performed at 65-95°C at the end of the PCR run. PCR reaction mixture contains 10 µl of TB Green Premix Ex Taq II (Takara), 10 pM of forward and reverse primers and 2µl of diluted cDNA to a final volume of 20µl. NTC (no template control) was maintained in every run for all the reference genes. Ct-values were recorded and utilized further for analysis.

Data analysis: The measurement of expression stability of seven reference genes across 48 samples was conducted with the help of two statistical software geNorm and Normfinder. Ct value obtained by qRT-PCR was converted to relative expression level for each reference gene with the help of delta-Ct method which was then used in geNorm and Normfinder for further analysis. Reference genes were ranked using geNorm based on expression stability value (M value) (Vandesompele *et al*. 2002). NormFinder tool determines expression stability using inter- and intra- group variation in the expression of candidate reference genes (Andersen *et al*. 2004).

RESULTS AND DISCUSSION

Amplification specificity and primer efficiency analysis of candidate reference genes: PCR amplification specificity of seven reference genes was evaluated in *S. alba* using melting curve analysis following qRT-PCR. Melting curve analysis showed single amplification with no primer dimer determined with a single peak for all the reference genes under investigation. In *S. alba* the linear R^2 value and PCR efficiency ranges from 0.9385 to 0.9942 and 96.62% to 103.02% respectively (Table 1). In order to obtain dependable results for qRT-PCR analysis, accurate normalization with several reference genes is essential. Primer specificity is a primary requirement for an accurate qRT-PCR analysis and for an ideal condition each primer efficiency should be close to 100%. Although it may vary from 95-105%.

Expression profiling of reference genes: In *S. alba* a wide Ct-value range was observed implicating a diverse expression pattern under each experimental condition (Fig 1). TUB1A was the most abundant reference gene (mean Ct-19.40) of the set whereas, PP2A exhibited lowest abundant expression (Mean Ct-25.68) across all the samples. Reference genes TIPS41 showed lowest gene expression variation (Coefficient of variation, CV of 5.39%) whereas the traditional housekeeping gene EF1A showed highest variation in gene expression (CV-8.26%) across all the samples. It was observed in our experiment that the novel reference gene exhibited lower gene expression variation as compared to traditional genes. These results indicated

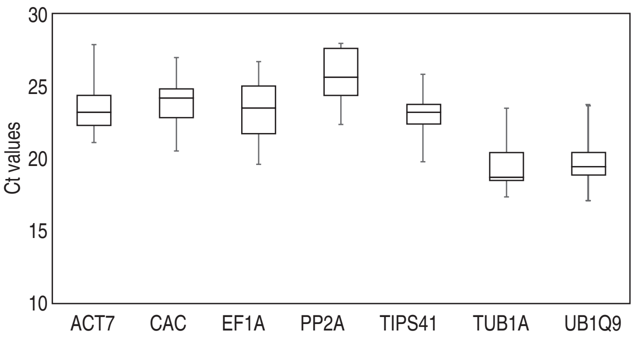


Fig 1 Expression levels of seven candidate reference genes.

Table 1 Description of the seven candidate reference genes adopted in the study

Gene symbol	Gene name	Accession number	Primers sequences 5'-3'	Amplicon length (bp)	T_m(°C) F/R	E (%)	R^2
ACT7	Actin 7	NM_121018	F-GGAATCGCTGACCGTATGAG R-ACCCTCCAATCCAGACACTG	109	60.3/60.0	99.23	0.9942
CAC	Clathrin adaptor complex	At5G46630	F-TTGAAGTTGGGGTTGAATGA R-AACAGTCTTCTCGGAGTTGAATC	150	58.9/58.9	96.62	0.9385
EF1A	Elongation factor 1 alpha	At1g07940	F-CCCTCCGTCTACCACTTCAG R-CACAACCATACCAGGCTTGA	101	59.7/59.6	100.36	0.9503
PP2A	Protein phosphatase 2A	At1g25490	F GTCAACAATCCGCACTACCTACA R-CAACCACGACGGGAAGAAAC	111	61.3/61.8	100.35	0.9833
TIPS41	Tonoplastic intrinsic protein 41	At4G34270	F-GGTTGAGAGAGACGAGAATGC R-ACTGGATACCCTTTCGCAGA	118	59.7/59.5	100.07	0.9729
TUA	Alpha tubulin	NM_121982	F-ACTTGGCTTGCTGTTTGATG R-CAGTTGGTGGCTGGTAGTTG	159	59.2/59.0	103.02	0.9709
UBQ9	Ubiquitin 9	At4g27960	F-CATCTTGAAGGAGCAGTGGA R-CAGTGGACTCGTACTTGTTCTTG	152	59.0/59.0	101.94	0.9905
PDF1.2	Defensin	AY133787.1	F- AGTCAAGTGGGACATGGTCA R-CTAACATGGGAAGTAGCAGATACAC	137	58.9/58.4	99.45	0.9867

* E- Primer efficiency

that none of the selected candidate reference gene project a near constant expression level and it is necessary to evaluate and select one or more stable reference gene(s) for accurate normalization under a defined set of experimental.

Solid line in the middle of the boxes represents median, and the boxes represents 25th and 75th percentile. Whiskers indicate the maximum and minimum values.

Gene expression stability and ranking of reference genes in S. alba: Expression stability of reference genes was identified in leaf samples belonging to following groups: Hormone treatment (SA, JA and ABA), wounding, *A. brassicae* infection and total (all samples taken together). Based on geNorm analysis, across all the samples of *S. alba* TIPS41 (M value-0.3023) and CAC (M value-0.3206) were the two most stable reference genes. Ranking of all the reference genes analyzed in their respective treatment groups and in total samples is depicted in Fig 2. For example, in hormone-treated samples TIPS41 and PP2A exhibited most stable expression with an M-value of 0.3012 and 0.3229 respectively. Expression data was also analyzed using NormFinder algorithm to further validate the geNorm analysis results. As depicted in Table 2, Ranking of reference genes generated by NormFinder across all the sample sets were found largely similar to the ranking given by geNorm program with minor differences. In hormone-treated samples, both the programs identified TIPS41, PP2A and CAC as three best reference genes therefore, TIPS41 and PP2A can be adopted as combination in the accurate normalization of hormone treated samples of *S. alba*. In wounded samples TIPS41, UBQ9 and PP2A were ranked as top three stable genes by both the programs. According to these results we recommend TIPS41and UBQ9 as the two most stable reference gene for wounded samples of *S. alba.* Contrastingly, in *A. brassicae* inoculated sample set, geNorm selected PP2A, UBQ9 and TUB1A as three best reference genes whereas, NormFinder suggested UBQ9, PP2A and TIPS41 as three most stable genes. Based on the algorithm variation in two program we recommend to

Table 2 Expression stability of seven reference gene and their ranking order calculated by NormFinder

Rank	All samples		Hormone		Wounding		*A. brassicae*	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	PP2A	0.244	PP2A	0.209	TIPS41	0.136	UBQ9	0.124
2	TIPS41	0.255	CAC	0.246	UBQ9	0.147	PP2A	0.137
3	CAC	0.295	TIPS41	0.288	PP2A	0.239	TIPS41	0.156
4	UBQ9	0.306	UBQ9	0.291	CAC	0.347	TUB1A	0.174
5	EF1A	0.360	EF1A	0.402	ACT7	0.397	ACT7	0.229
6	ACT7	0.391	TUB1A	0.447	TUB1A	0.558	CAC	0.252
7	TUB1A	0.449	ACT7	0.466	EF1A	0.618	EF1A	0.333

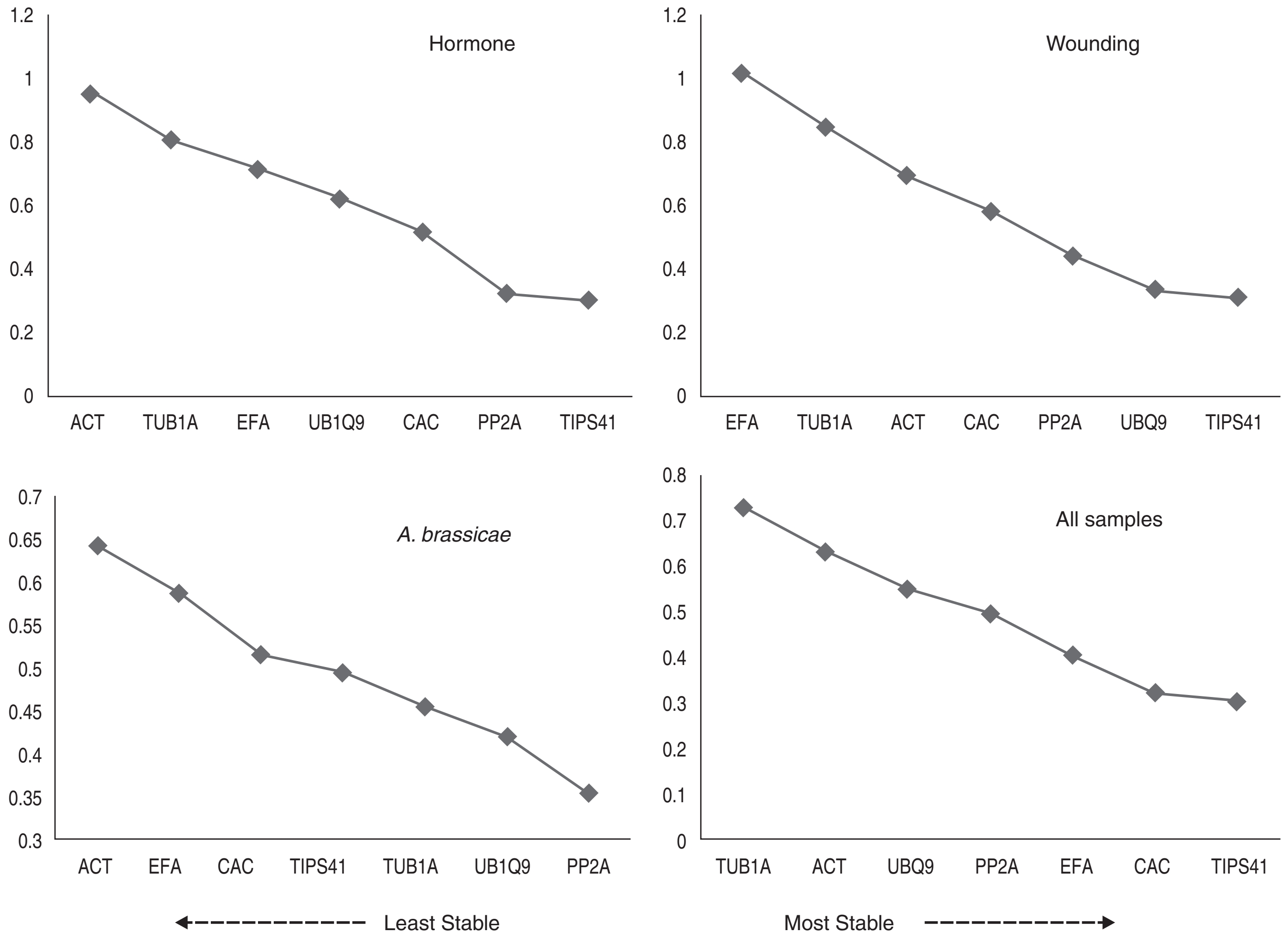


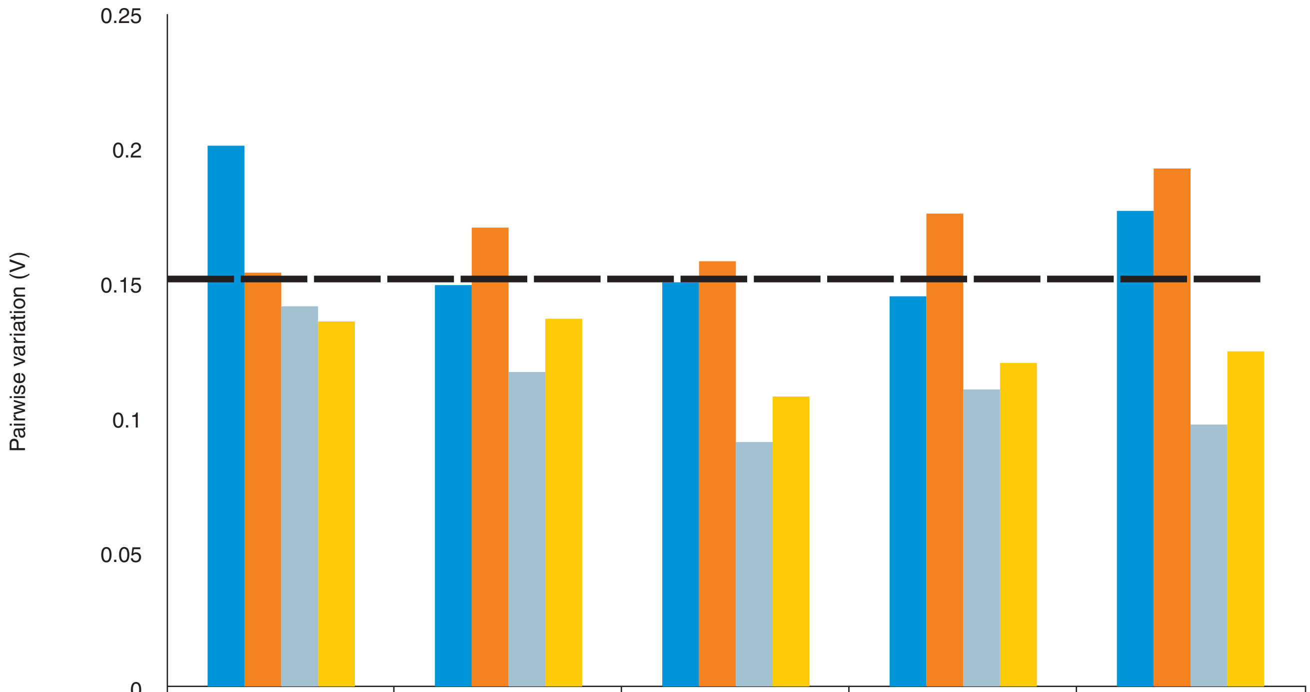
Fig 2 Average expression stability and ranking of seven reference genes in *S. alba* predicted by geNorm (lower M-value depict stable expression and *vice versa*).

adopt common genes PP2A and UBQ9 as reference genes in the experiment conducted on *A. brassicae* infected samples of *S. alba*.

Minimum number of candidate reference genes for accurate normalization in both the genotypes: geNorm program also identifies the minimum number of reference gene that must be used for accurate normalization of a given experimental set. Vandesomple *et al.* (2002) recommended cutoff value of 0.15, beyond which adding new genes has no effect on expression stability value. As shown in Fig 3, pairwise variation V2/3 of all the sample sets were found to be less than 0.15 therefore, two most stable reference genes are sufficient to perform an accurate normalization of qRT-PCR analysis except for hormone treated sample set where the V3/4 value was less than 0.15 which means three genes are required for normalization.

Reference gene validation in S. alba: To validate the reference genes confirmed above, relative expression of PDF1.2 was measured in JA-treated samples of *S. alba* at different time intervals after treatment. PDF1.2 is a reported marker gene for JA-induced signaling and it has been found to induce resistance in plants towards necrotrophic pathogen like *A. brassicae* (Lay and Anderson 2005, Rowe *et al*. 2010). For qRT-PCR analysis one most stable and one least stable reference genes were used and their impact on overall expression level was observed. As shown in Fig 4, in JA-treated samples, PDF1.2 showed induction at 3 h and it raises up to 6 h and gradually return to control level with time. When the expression level of PDF1.2 was calculated using most stable reference gene (TIPS41) highest induction of 4.5 fold was observed at 6 h after treatment. Whereas, when the expression level of PDF1.2 was evaluated with the least stable reference gene (ACT7), an overestimation of the expression level was observed where approximately six-fold higher expression of PDF1.2 was noticed at 6 h after treatment. These results established that using an inappropriate reference gene highly influences the accuracy of the expression analysis. The expression pattern analysis of PDF1.2 in JA-treated samples emphasized on the importance of choosing a suitable reference gene. When different reference genes were adopted for gene expression analysis of PDF1.2, it is clearly evident that using a unstable reference gene causes a misinterpretation of the gene expression patterns leading to overestimation of the fold change in treated samples with respect to control samples (Fig 4).

Relative quantification of PDF1.2 in JA-treated samples of *S. alba* using most stable (TIPS41) and least stable



	V2/3	V3/4	V4/5	V5/6	V6/7
🟦 Harmone	0.201855941	0.150652903	0.152109539	0.145762427	0.178039648
🟧 Wounding	0.154197016	0.171356275	0.15848192	0.176673273	0.193354473
▨ *A. brassicae*	0.141630241	0.117072981	0.091196413	0.111253067	0.097568828
🟨 Total	0.136962805	0.137870568	0.108182235	0.120897877	0.125126525

Fig 3 Determination of optimum number of reference genes for an accurate normalization.

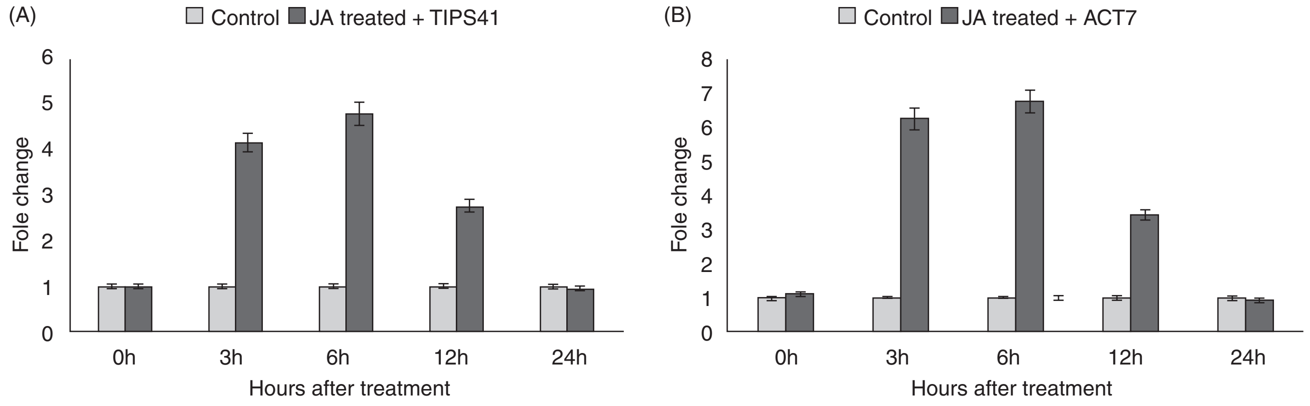


Fig 4 Validation of the identified reference genes.

(ACT7) reference gene

In the present study, we evaluated the expression stability of 7 candidate reference genes across diverse sample sets of *S. alba* in order to identify suitable reference gene(s) for normalization of gene expression analysis. geNorm and NormFinder analysis recognized TIPS41 and PP2A as the two most stable reference genes across 48 samples of *S. alba*. However, suitable reference gene for each treatment is recommended accordingly. Expression pattern analysis of PDF1.2 in JA-treated samples emphasized on the importance of selecting suitable reference gene for accurate qRT-PCR analysis. We conclude that the results summarized in our investigation will facilitate accurate quantification of gene expression in *S. alba* under various stress condition.

REFERENCES

Andersen C L, Jensen J L, and Ørntoft T F. 2004. Normalization of real-time quantitative reverse transcription-PCR Data : A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research* **64**(15): 5245-50.

Chandna R, Augustine R and Bisht N C. 2012. Evaluation of candidate reference genes for gene expression normalization in *Brassica juncea* using real time quantitative RT-PCR. **7**(5): https://doi.org/10.1371/journal.pone.0036918.

Czechowski T, Stitt M, Altmann T and Udvardi M K. 2005. Genome-Wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physisology*. **139**: 5-17.

Duan M, Wang J, Zhang X, Yang H and Wang H. 2017. Identification of optimal reference genes for Expression Analysis in radish (*Raphanus sativus* L.) and its relatives based on expression stability. *Frontiers in Plant Science*. **8**.1605: 10.3389/fpls.2017.01605

Gachon C, Mingam A and Charrier B. 2004. Real-time PCR: what relevance to plant studies? *Journal of Experimental Botany* **55402**: 1445-1454.

Gutierrez L, Mauriat M, Guénin S, Pelloux J, Lefebvre J F, Louvet R and Van Wuytswinkel O. 2008. The lack of a systematic validation of reference genes: A serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. *Plant Biotechnology Journal* **6**(6): 609-618.

Jain M, Nijhawan A, Tyagi A K and Khurana J P. 2006. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. *Biochemical and Biophysical Research Communications*. **345**(2): 646-651.

Kundu A, Patel A and Pal A. 2013. Defining reference genes for qPCR normalization to study biotic and abiotic stress responses in *Vigna mungo*. *Plant Cell Reports*. **32**(10): 1647-1658.

Lay F T and Anderson M A. 2005. *Defensins* - Components of the innate immune system in plants. *Current Protein and Peptide Science*. **6**(1): 85-101.

Pieterse M J, Does D V D, Zamioudis C, Leon-reyes A and Wees S C M V. 2012. Hormonal modulation of plant immunity. *Annual Reviews of Cell and Developmental Biology.* 10.1146/annurev-cellbio-092910-154055.

Rowe H C, Walley J W, Corwin J, Chan E K, Dehesh K and Kliebenstein D J. 2010. Deficiencies in jasmonate-mediated plant defense reveal quantitative variation in *Botrytis cinerea* pathogenesis. *PLoS Pathogen*. **6**(4). https://doi.org/10.1371/journal.ppat.1000861.

Schmidt G W and Delaney S K. 2010. Stable internal reference genes for normalization of real-time RT-PCR in tobacco (*Nicotiana tabacum*) during development and abiotic stress. *Molecular Genetics and Genomics* **283**(3): 233-241.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth B C, Remm M and Rozen S G. 2012. Primer3-new capabilities and interfaces. *Nucleic Acids Research*. **40**(15): 1-12.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A and Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology.* **3**(7): research0034.1-0034.11.

Yang H, Liu J, Huang S, Guo T, Deng L and Hua W. 2014. Selection and evaluation of novel reference genes for quantitative reverse transcription PCR (qRT-PCR) based on genome and transcriptome data in *Brassica napus* L. *Gene*. **538**(1): 113-122.