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Evaluation of reference genes for gene expression studies in radish (*Raphanus sativus* L.) using quantitative real-time PCR

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ABSTRACT

Real-time quantitative reverse transcription PCR (RT-qPCR) is a rapid and reliable method for gene expression studies. Normalization based on reference genes can increase the reliability of this technique; however, recent studies have shown that almost no single reference gene is universal for all possible experimental conditions. In this study, eight frequently used reference genes were investigated, including Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Actin2/7 (*ACT*), Tubulin alpha-5 (*TUA*), Tubulin beta-1 (*TUB*), 18S ribosomal RNA (*18SrRNA*), RNA polymerase-II transcription factor (*RPII*), Elongation factor 1-b (*EF-1b*) and Translation elongation factor 2 (*TEF2*). Expression stability of candidate reference genes was examined across 27 radish samples, representing a range of tissue types, cultivars, photoperiodic and vernalization treatments, and developmental stages. The eight genes in these sample pools displayed a wide range of Ct values and were variably expressed. Two statistical software packages, geNorm and NormFinder showed that *TEF2*, *RPII* and *ACT* appeared to be relatively stable and therefore the most suitable for use as reference genes. These results facilitate selection of desirable reference genes for accurate gene expression studies in radish.

1. Introduction

RT-qPCR is a sensitive and accurate technique known for specificity, reproducibility and broad dynamic range, and has become the method of choice for gene expression analysis [1]. Although RT-qPCR has many advantages, reliable results are dependent on the accuracy and precision of experimental conditions, including quantity of initial material, primer design and performance, reference gene selection, RNA extraction technique, cDNA synthesis efficiency, and statistical analysis methods [2]. Since reference genes are run and analyzed with the gene of interest, they are a frequently used method for normalization of RT-qPCR data, thus avoiding possible errors caused by variations within experimental conditions [3].

Ideal reference genes are constitutively expressed, involved in basic cellular functions and life processes among different tissues and developmental stages. Moreover, reliable reference genes should be unaffected by experimental conditions [4,5] and have a transcript level similar to the target gene [6]. Nevertheless, recent studies have indicated that stability among some commonly-used reference genes is relative, with no single gene having a constant stable expression under all conditions [7]. This variation in expression stability may be caused by involvement of the reference gene in additional, non-constitutive cellular processes [8]. Thus, careful selection of a reference gene is critical to proper function within gene expression studies, and to accurate data analysis and conclusions [9]. To ensure appropriate normalization, a systematic validation of reference genes at specific experimental conditions has become vital to effective use of RT-qPCR [5].

Recently, studies validating the importance of reference genes have been performed on plants including *Arabidopsis thaliana*, potato, soybean, grape, tomato, chicory, tobacco, cucumber, litchi, perennial ryegrass, peanut, flax and zucchini [3,4,7,10–20]. To evaluate the suitability of the reference gene in a range of samples, many statistical analysis procedures have been developed, including geNorm and NormFinder [21,22]. To date, however, no related study has been reported in radish (*Raphanus sativus* L.).

Radish (*R. sativus* L.) originated in China and is an important root vegetable crop. Proper regulation of flowering time would improve production and quality; however, although considerable research has focused on understanding vernalization and photoperiodic control of flowering, these mechanisms in radish are not yet well understood. A more complete grasp of vernalization and flowering may require elucidation of key genes in the biochemical pathways controlling these traits. Precise gene expression analysis would provide a powerful and valuable approach for understanding the

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biological and molecular mechanisms in growth and development, as well as signal transduction and metabolism.

In this study, expression profiles of eight reference genes (*GAPDH*, *ACT*, *TUA*, *TUB*, *18SrRNA*, *RPII*, *EF-1b* and *TEF2*) were evaluated across 27 radish samples. The expression patterns of these reference genes were tested in a range of tissue types, cultivars, photoperiodic and vernalization treatments, and developmental stages, to identify a stable reference gene for gene expression studies in radish.

2. Materials and methods

2.1. Plant materials

Five radish advanced inbred lines were used which varied in the bolting character (NauDY13, NauSH, NauZQH, NauXBC are early bolting and NauXXXS is late bolting). Germinated seeds were vernalized and sown in the greenhouse. Seedlings at the 6-leaf stage were transferred to a growth chamber (28/16 °C day/night). For subgroups of different cultivars, leaves of NauDY13, NauSH, NauZQH, NauXBC and NauXXXS were collected at the vegetative stage in the MD (12 h light/12 h dark) condition. For subgroups of varied vernalization treatments, leaves of NauDY13 were collected in the LD (16 h light/8 h dark) treatment of the reproductive stage and vernalized at 2–4 °C for 0, 10, 20, 30, 40 d, respectively. For subgroups of different tissue types, samples of root, leaf, stem, shoot, petiole, flower and bud were taken from NauXXXS in the MD condition at flowering stage. For subgroups of different photoperiodic treatment, leaves of NauXXXS vernalized for 20 d in the vegetative stage with photoperiodic treatments were collected from LD/MD/SD (8 h light/16 h dark) treatments. For subgroups of different developmental stages, the cotyledon, the first true leaf, the second leaf, the fourth leaf, the sixth leaf, the eighth leaf and the flower stalk leaf of NauXXXS, which were vernalized for 40 d and grown under the MD condition, were collected. All 27 samples were immediately frozen in liquid nitrogen and stored at −80 °C for further use.

2.2. Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted from different tissues with a Simply P total RNA extraction kit (BioFlux, China) according to the manufacturer's procedure. Three replicates of RNA extraction were conducted. Genomic DNA was eliminated from total RNA with RNase-free DNase I, and first-strand cDNA was synthesized with the M-MLV (RNase H^-) (TaKaRa, Japan) and oligo-dT primers.

2.3. RT-qPCR primer design, selection, and amplified product verification

Primers were designed using Beacon Designer 7.0 (Premier Biosoft International, USA) for RT-qPCR. To ensure optimal polymerization efficiency and reduce the impact of RNA integrity on gene expression in RT-qPCR, primers were selected with a Tm of 58–62 °C, length of 18–24 bp, and GC content of 45–55%. Primers were designed to amplify products within the range of 101–155 bp (Table 2). Specificity of the amplification product for each primer pair was verified by the presence of a single band of the expected size, using agarose gel electrophoresis, and by the presence of a single peak in RT-qPCR melting curve products (Fig. 1).

2.4. Real-time quantitative RT-PCR

RT-qPCR was conducted on a MyiQ Real-Time PCR Detection System (Bio-Rad) using the SYBR Green Master ROX (Roche, Japan) following the manufacturer's instructions. Each reaction was prepared in a total volume of 20 μl containing 10 μl SYBR Green Mix, 1.5 μl diluted cDNA and 0.2 μM of each primer. Reactions were subjected to an initial denaturation step of 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 58 °C for 30 s and 72 °C for 10 s. A melting curve analysis was performed at the end of the PCR run over the range 60–95 °C, increasing the temperature stepwise by 0.5 °C every 10 s. Each reaction contained a reverse transcription negative control to ensure there was no potential genomic DNA contamination. A non-template control was conducted to detect possible reagent contamination. Three independent biological duplications were performed for all reference genes, and each sample was prepared in triplicate.

2.5. Data analysis

The Ct value of each reference gene was used to compare expression levels in all different radish samples. PCR efficiency was derived from amplification plots using the program LinRegPCR [23]. Results were imported into Excel, and geNorm and NormFinder were used to determine normalization effectiveness of each reference gene among all 27 samples. The geNorm software provides a stability measure (M) and excludes the least stable gene, using a stepwise method and creating a line graph to show stability of the selected genes [21]. NormFinder uses an ANOVA-based model to evaluate intra-group and inter-group variation, providing a direct measure of the variation [22].

Table 1
Radish reference genes used for RT-qPCR.

Name[a]	Radish EST database accession number	Arabidopsis homolog locus[b]	Arabidopsis locus description	Function	Identities (%)
GAPDH	FY441507	AT1G13440	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	100.0
Actin2/7	FY430005	AT5G09810	Actin 2/7	Structural constituent of cytoskeleton	95.5
TUA	FY452411	AT5G19780	Tubulin alpha-5	Cytoskeleton structural protein	98.2
TUB	FY449777	AT1G75780	Tubulin beta-1	Unidimensional cell growth, response to light stimulus	93.5
18SrRNA	FY434552	AT3G41768	18S ribosomal RNA	Cytosolic small ribosomal subunit, translation	99.3
RPII	FY438464	AT2G15430	RNA polymerase-II transcription factor	DNA-directed RNA polymerase activity, DNA binding	99.3
EF-1b	EY936648	AT5G19510	Elongation factor 1-beta	Translational elongation	98.7
TEF2	FY430003	AT1G56070	Translation elongation factor 2	Translation factor activity, nucleic acid binding	97.7

[a] All radish ESTs were named based on similarity to *Arabidopsis* proteins determined by BLASTX.
[b] The closest *Arabidopsis* homolog identified using TAIRBLAST.

Table 2
Primer sequences and amplicon characteristics for the reference genes.

Name	Forward Primer Sequence [5′-3′]	Reverse Primer Sequence [5′-3′]	Amplicon Size (bp)	Product™ (°C)[a]	RT-qPCR Efficiency[b]	R^{2b}
GAPDH	GAAATCAAGAAGGCTATCAAGGAG	TTGTCACCAACGAAGTCAGT	101	80.2	1.866	0.998
Actin2/7	GCATCACACTTTCTACAAC	CCTGGATAGCAACATACAT	155	79.3	2.040	0.998
TUA	TTCCCTATCCTCGCATCCATTTCA	CCTCGGGTCACACTTAGCCATCA	146	79.5	1.876	0.999
TUB	GTCCGGTGCTGGTAATAACTGG	GTGGCATACTTGAAACCCTTGAA	130	79.0	1.825	0.999
18SrRNA	TACCGTCCTAGTCTCAACCATAA	TTTCAGCCTTGCGACCATAC	130	79.2	1.955	1.000
RPII	ATCACGCTAAATGGTCTCCT	GCTGCTCTCAATCAAGTCAATC	122	76.0	2.009	0.999
EF-1b	GCTGCTAAGAAACATACCAAG	AACCAGGCATCTGAACACTA	133	76.4	1.885	0.999
TEF_2	AAGAAGATTTGGGCGTTTGG	CCAGCAACAACAGAATCCTT	107	77.8	1.916	0.998

[a] The melting temperature of specific PCR product was calculated by MyiQ Real-Time PCR Detection System (Bio-Rad).
[b] The RT-qPCR efficiency and correlation coefficients (R^2) were determined with LinRegPCR software.

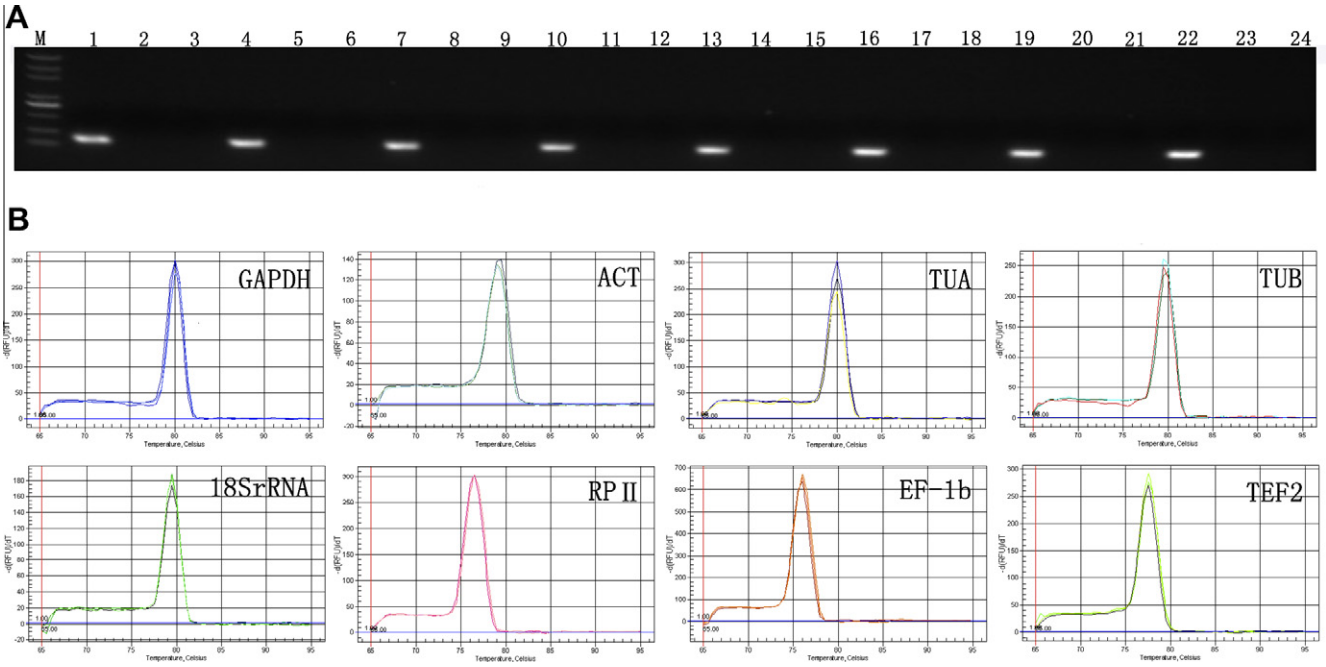


Fig. 1. Specificity of the amplification products. (A) Lanes 1, 4, 7, 10, 13, 16, 19 and 22 were *GAPDH*, *ACTIN*, *TUA*, *TUB*, *18SrRNA*, *RPII*, *EF-1b*, *TEF2*, respectively. Lanes 2, 5, 8, 11, 14, 17, 20 and 23 were control without reverse transcriptase; Lanes 3, 6, 9, 12, 15, 18, 21 and 24 were control with no template. M: DL2000 plus DNA ladder. (B) Melt curves analysis generated for amplicons of each of the eight reference genes.

3. Results

3.1. Primer selection and amplified product verification

To select the best stable reference genes for radish gene expression studies, RT-qPCR primers were designed for eight widely-used reference genes (Table 1). Amplification specificity methods, including a single band in gel electrophoresis (Fig. 1A) and presence of a single peak in RT-qPCR melt curve products (Fig. 1B) indicated that there were no primer-dimers and other non-specific amplification products. Additionally, no RT-qPCR detection signals were collected on reactions without template.

3.2. Ct value analysis

To obtain reliable results, all PCR assays were conducted in triplicate and the mean was used for analysis. In this study, Ct values of the eight reference genes showed a relatively wide range, from 15.58 in *18SrRNA* to 37.48 in *TUA*. Most values ranged from 21.2 to 32.3 (Fig. 2). Threshold fluorescence for *18SrRNA* was reached in only 15.58 cycles, indicating a high level of expression compared with other reference genes. Overall, the wide expression range

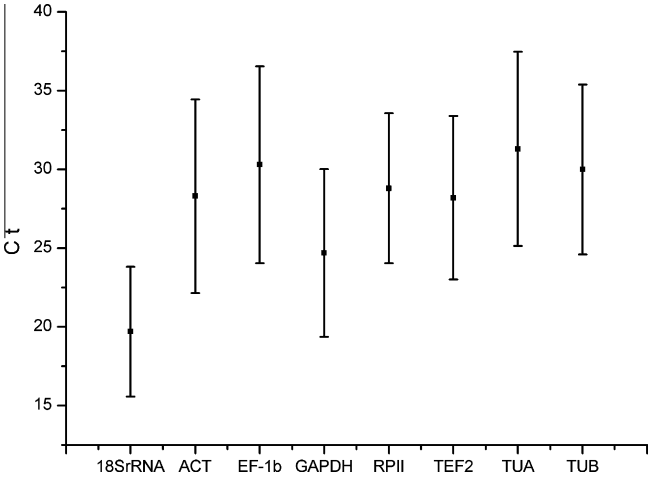


Fig. 2. Absolute cycle threshold values (Ct) for eight reference genes over 27 cDNA radish samples in RT-qPCR.

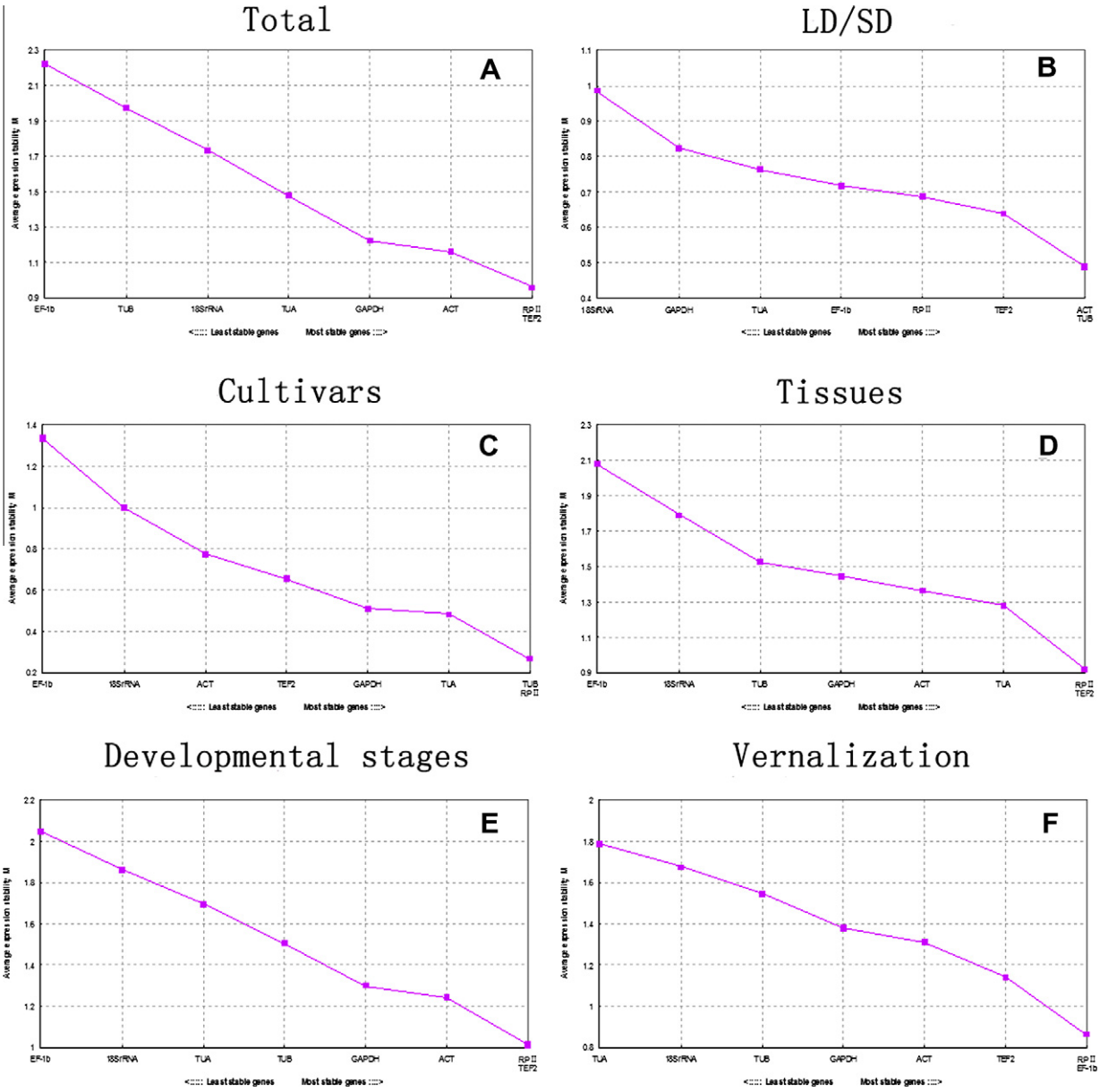


Fig. 3. Average gene expression stability (M) used to ranking internal control genes within different treatment groups. Calculations were performed by geNorm.

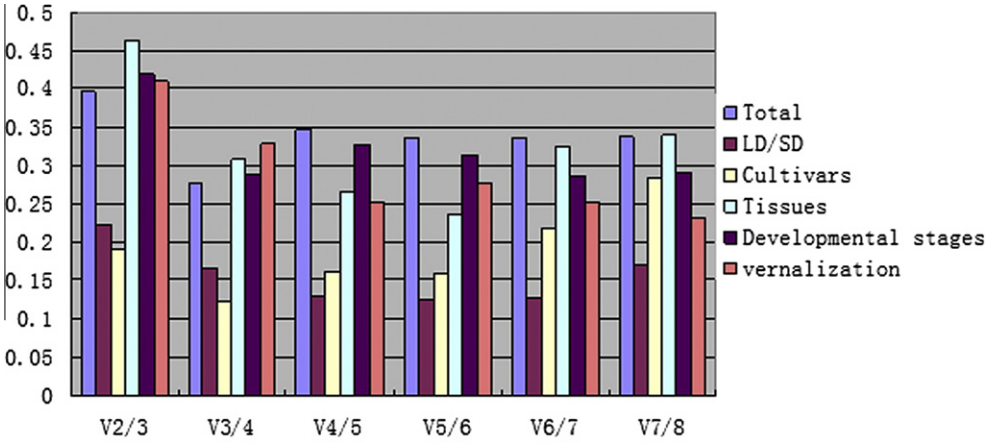
indicated that no single gene had a stable and constant expression under all experimental conditions.

3.3. GeNorm analysis

The geNorm analysis ranked reference genes according to expression stability (*M* value), facilitating selection of an optimal pair of reference genes from a group of candidate genes in different treatment samples under different conditions. The gene with the lowest *M* value is thought to be the most stably expressed. When all 27 samples were taken together, *RPII* and *TEF2* were the most stably expressed and *EF-1b* was least stably expressed (Fig. 3A). Similar results were obtained under experimental conditions in different tissues and developmental stages (Fig. 3D and E). *TUB* and *RPII* were stably expressed in subgroup for different radish lines, with *EF-1b* being the least stable (Fig. 3C). In the photoperiodic treatment, *ACT* and *TUB* were the most stable, while *18SrRNA* was the most variable (Fig. 3B). Among different vernalization conditions, the lowest *M* values were obtained for *RPII* and *EF-1b*, and the highest for *TUA* (Fig. 3F). Overall, *RPII* and *TEF2* were expressed stably, while *EF-1b* and *18SrRNA* were expressed much more variably than the other reference genes.

To determine the necessity of adding additional reference genes, and the optimal number of genes required for effective normalization, pairwise variation Vn/Vn + 1 between sequential normalization factors NF (NFn and NFn + 1) were introduced. Effective numbers varied for different subgroups. For example, using a cut-off value of 0.15 in the experimental condition of different photoperiodic treatments, four reference genes should be selected (V4/5 < 0.15), while for different cultivars comparison, three reference genes (*TUA*, *TUB* and *RPII*) were sufficient (Fig. 4).

However, 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 [15]. Other studies have shown a higher V number for the species under consideration [24]. In this study, pairwise variation Vn/Vn + 1 was above 0.15 for the pooled sample analysis and for different subgroups. Considering these values, we suggest using the three top ranked reference genes, which



	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
Total	0.396	0.277	0.346	0.335	0.335	0.337
LD/SD	0.224	0.167	0.128	0.124	0.126	0.171
Cultivars	0.192	0.121	0.163	0.158	0.219	0.284
Tissues	0.465	0.308	0.266	0.237	0.324	0.339
Developmental stages	0.419	0.288	0.327	0.312	0.286	0.29
Vernalization	0.409	0.329	0.252	0.277	0.252	0.232

Fig. 4. Determination of the optimal number of reference genes for normalization by geNorm.

Table 3
Ranking of eight candidate reference genes in order of their expression stability calculated by NormFinder.

Rank	Total		LD/SD		Cultivars		Tissues		Developmental stages		Vernalization	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*RPII*	0.016	*TUB*	0.012	*RPII*	0.005	*RPII*	0.023	*RPII*	0.020	*RPII*	0.018
2	*TEF2*	0.033	*TEF2*	0.014	*TUB*	0.005	*TEF2*	0.029	*ACTIN*	0.029	*EF-1b*	0.031
3	*ACTIN*	0.034	*ACTIN*	0.017	*TUA*	0.013	*ACTIN*	0.036	*TEF2*	0.034	*TEF2*	0.035
4	*GAPDH*	0.048	*EF-1b*	0.019	*GAPDH*	0.015	*TUA*	0.045	*GAPDH*	0.048	*ACTIN*	0.035
5	*18SrRNA*	0.064	*RPII*	0.022	*TEF2*	0.036	*TUB*	0.049	*TUA*	0.058	*GAPDH*	0.050
6	*TUB*	0.057	*TUA*	0.024	*ACTIN*	0.054	*GAPDH*	0.055	*TUB*	0.061	*TUB*	0.055
7	*TUA*	0.059	*GAPDH*	0.035	*EF-1b*	0.058	*18SrRNA*	0.068	*18SrRNA*	0.062	*18SrRNA*	0.058
8	*EF-1b*	0.086	*18SrRNA*	0.049	*18SrRNA*	0.067	*EF-1b*	0.088	*EF-1b*	0.074	*TUA*	0.060

would improve accuracy and reliability compared with using a single reference gene.

3.4. NormFinder analysis

NormFinder, another useful algorithm, provides not only a mathematical model of gene expression, but also a stability value for each gene between each sample subgroup. More stable gene expression is indicated by lower average expression stability values. Three genes, *RPII*, *TEF2* and *ACT*, showed remarkable expression stability and were always highly ranked, while *EF-1b* and *18SrRNA* exhibited unstable expression profiles and were always among the least stable reference genes (Table 3).

When NormFinder was used to analyze the five subgroups separately, *TUB* was calculated to be the most stable single gene for different photoperiodic treatments, with a stability value of 0.012, while *18SrRNA* was the least stable. When evaluating different tissue types and developmental stages, *RPII* was ranked as most stable, and *EF-1b* as least stable. *RPII* remained the most reliable reference gene in subgroups for different cultivars and vernalization treatment conditions, while *18SrRNA* and *TUA* replaced *EF-1b* as the most variable.

Results from the geNorm analysis were similar, with *RPII* and *TEF2* in most cases considered the most stable reference genes in radish. *EF-1b* and *18SrRNA* were considered the least stable under different treatment conditions.

4. Discussion

Reference genes are important for quantifying gene expression. Ideally, reference genes should be expressed at a constant level throughout the life of the plant, and not influenced by experimental conditions [9,10]. Therefore, it is necessary to validate expression stability of selected reference genes under specific conditions. The present study is the first comprehensive survey on identifying appropriate reference genes for RT-qPCR analysis in radish.

In this study, two statistical algorithms, geNorm and NormFinder, were applied. GeNorm relies on the assumption that the genes being analyzed are not regulated together, which is problematic in the case of an unsuitable choice of reference gene pairs [21]. The software also provides useful information regarding an optimal number of reference genes. Co-regulated genes are always similarly ranked, although reference genes with the same function are not always top-ranked [10]. Considering that reference genes may affect other cellular systems and decrease the value of the function-based predictions of co-regulation, NormFinder ranks the set of candidate reference genes according to the least of their estimated intra- and inter-group variations [15].

There is some discrepancy between results from geNorm and NormFinder, probably because the two programs were based on different algorithms and analytical procedures [21,22]. Since *RPII*, *TEF2* and *ACT* were highly ranked by geNorm and NormFinder in all radish samples, these would be considered reliable reference genes. Within the five subgroups, *RPII* was ranked first with the exception of the subgroup for photoperiodic treatment. *TEF2* and *ACT* were ranked in the top four except in the subgroup of different cultivars. It is well known that *RPII* and *TEF2* are needed for elongation and mRNA transcription in eukaryotes, respectively [25], which may explain why they remained continuously expressed and showed minimal variations. This stability was also found in previous studies in peach [10]. Thus, *RPII* and *TEF2* could serve as ideal reference genes. *ACT* was a major component of cytoplasmic microfilaments in eukaryotic cells [10] and has been reported to be variable in *Arabidopsis* but stable in soybean [12]. In this study, *ACT* was one of the most stable reference genes, confirming the necessity to evaluate reference genes in a specific experimental setting.

On the other hand, *18SrRNA* and *EF-1b* performed poorly in most cases, indicating that in the current experimental conditions these two genes were not consistently and steadily expressed and should not be used as reference genes. Previously-reported disadvantages of *18SrRNA* include its high abundance, making it difficult to accurately subtract the standard value in RT-qPCR data analysis, and its limitation in using oligo-dT as primers and mRNA as the template for reverse transcription reaction [26]. In this study, *18SrRNA* displayed poor stability, concurring with a previous report in broomrape tissues [27]. Expression of *EF-1b*, which functions in translational elongation, was stable in soybean and potato [7,12]. However, *EF-1b* proved to be one of the least stable candidate genes in radish in this study, concurring with previous results in tomato [14].

Remaining candidate genes, *GAPDH*, *TUA* and *TUB*, displayed unacceptably variable expression patterns and were not considered reliable reference genes. *GAPDH* may not only act as a component of the glycolytic pathway, but also participate in other life processes [10]. *TUB* was reported to perform with poor stability in grape, potato and soybean [13]. In this study, *TUB* showed unstable expression patterns in most cases, although it showed high expression stability in subgroups comparing different cultivars and photoperiodic treatment conditions, again indicating that stability of a reference gene may vary under different conditions.

Radish is a typical long-day plant and requires vernalization to promote bolting and flowering. The effects of the regulator genes on bolting and flowering have not yet been explored. The present research on evaluating reference genes for bolting and flowering investigation would provide normalization for the expression of target genes in radish. Among the eight candidate genes, *TEF2*, *RPII* and *ACT* were stable under different experimental conditions, and *EF-1b* and *18SrRNA* were unstable in most cases. The combination of two or more stable genes would significantly improve accuracy in expression profiling among multiple samples. Application of selected reference genes provides a valuable approach for better understanding the molecular mechanisms of bolting and flowering in radish.

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