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Development and Assessment of Reference Genes for qPCR of *Malus transitoria* under Drought Stress

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ABSTRACT: The use of a stable reference gene is fundamental for achieving reliable quantitative qRT-PCR (qPCR) results. Developing and evaluating the stability of reference genes is necessary for studying the molecular mechanisms of *M. transitoria* in response to drought stress. In this study, 18 candidate reference genes were selected from transcriptome sequencing data of *M. transitoria* according to their FPKM values under different drought stress degrees. Cluster-23533.34641 was identified as the most stable reference gene for *M. transitoria* under drought stress based on qPCR results and combined analysis of Genorm, NormFinder, BestKeeper, and Delta Ct algorithms. The reference genes identified in this research offer improved accuracy for quantifying target gene expression in both *M. transitoria* and *Malus* species under drought stress. This study could provide insights into the drought stress-related functional gene or factor in *M. transitoria*, even in *Malus* species.

KEYWORDS: Reference genes; *Malus transitoria*; drought stress; qPCR

1 Introduction

Malus transitoria, a native *Malus* species to China, is mostly located in higher elevations regions of arid and semi-arid regions of China, such as Qinghai-Tibetan Plateau, Shanxi, Sichuan, Gansu, and Inner Mongolia [1,2]. Based on its drought resistance performance, *M. transitoria* has been used as a tree species for afforestation and ecology restoration in arid areas, especially in the Qinghai-Tibetan Plateau. Our previous research showed, with climate change, the distribution area of *M. transitoria* would constantly migrate toward the north, and some populations have lost genetic diversity and showed a trend of shrinking in size [3,4], therefore, it is necessary to study the mechanism and find the key gene related *M. transitoria* cope with drought stress.

As a key factor in the study of gene function, the stability of the reference gene is influenced by development stage, stress degree, tissue types, and experimental conditions. Therefore, developing and evaluating reliable reference genes is a prerequisite in research on plant development and gene function.

In general, housekeeping genes are consistently expressed across all tissues and play a vital role in sustaining the essential cellular functions of plants, being used as a reference gene. In most of the fruit trees,



Ubiquitin extension (UBQ) [5], Actin isoform B (*ACTB*), 18S rRNA [6], *GAPDH*, *EF1*, *ACT* [7–9], *UBQ-I* [7], and others have been used as reference gene.

In the closely related species of *M. transitoria*, there are some reports about the validation of reference genes. For example, in apples, *EF-1α* and 18S rRNA were used as reference genes for research of the fruit quality during the fruit coloring period [10], *UBQ* was used as the reference gene in the different development stages of different genotype apples, and *ACTB* and *UBQ* were used in the different tissue [5]. *WD40*, *ACT*, and *GAPDH* were identified as the most reliable reference genes for apple peel [9], the suitability of phosphatase 2A (PP2A), ribosomal protein L2 (RPL2), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as reference genes were assessed across various apple tissue types and under diverse biotic stress conditions, but the transitional housekeeping gene, 18S ribosomal RNA (18S), β -tubulin (*TUB*) and ubiquitin (*UBQ*) were evaluated as the least stable genes [11]. The *MDH*, *SAND*, *THFS*, *TMp1*, and *WD40* were evaluated as the best reference genes to accurately normalize gene expression levels in various tissues of apple [12]. In various tissues and organs of loquat, RPL4, RPL18, HIS3, and TUA3 were identified as the most reliable reference genes [13]. In Herbaceous peonies, *EF-1α* demonstrated considerable stability as an internal reference gene when exposed to drought stress [14]. In Kiwifruit, *GAPDH* and *ACT1* are the most suitable reference genes during fruit development [15]. In other species, such as *Oryza sativa* L. under salt stress, *EIF1A* and *TIP* are the most suitable internal reference genes [16]. In *Brassica rapa* L., *EF1-α*, *UBC*, and *ACT* as the most suitable reference genes [17]. In *Kobresia littledalei*, *ACTIN* and *GAPDH* as the most suitable reference genes [18]. In *Luffa cylindrica*, *EF-1α* was the overall most stable and suitable reference gene [19].

Besides these traditional housekeeping genes, more and more reference genes were developed and assessed with sequencing and bio-information technology zooming. Five potential candidate reference genes were developed and evaluated under various abiotic or biotic stresses in apple roots. Based on the transcriptome sequencing, four potential genes that could be used in post-harvest and other apple gene expression experiments were developed and evaluated [20]. Combining mRNA-seq with qPCR, three potential reference genes are proposed that can be used as reference genes for qPCR in *Malus x domestica* (apple) [21]. *HISTONE 1* (*MdH1*) was evaluated as the most stable gene in experiments encompassing of apple post-harvest period based on the Transcriptional Profiling [22].

However, previous studies have shown that some housekeeping genes are expressed deferentially at different stages of plant development or under different stresses and conditions [23,24], which suggested that randomly selected reference genes may be inappropriate, leading to deviations or even incorrect results, and further affecting the accuracy of the conclusions [25]. As a result, choosing reference genes with stable expression based on the specific experimental needs is essential for obtaining reliable results.

At present, there are no reports about specific reference genes that have been developed for *M. transitoria*, especially for stressed samples. To correctly evaluate the expression of a gene that relates to drought resistance in *M. transitoria*, in this study, we developed and assessed the reference genes based on the transcriptome of drought-stressed samples. The stability of these candidate genes was assessed using four algorithms based on the result of qPCR amplification. These assessed internal reference genes could provide an important basis for improving the accuracy of qPCR in analyzing gene expression under drought stress in *M. transitoria*. This study could lay a solid foundation for future gene function studies of *M. transitoria* under drought stress conditions, and further afford useful tools in studying the mechanisms of tolerance in this species.

2 Materials and Methods

2.1 Plant Materials Collection

The experiment began on 9 July 2023, at the Forestry Experimental Base of Qinghai University in Qinghai Province. Five treatment groups were set up, with 1.5 kg of soil in the pots in each group. At the beginning, 200 mL of water was simultaneously watered in the 5 treatment groups, for the CK, keep the soil surface moist throughout the entire experimental process, for T1, watering 200 mL every three days, 200 mL every six days for T2, 200 mL every nine days for T3 and 200 mL every twelve days for T4. On the second day after the third round of watering in each treatment group, three biological replicates were taken from each treatment group. The samples were placed in liquid nitrogen and can only be used for subsequent experiments. The phenotype under drought stress is shown in Fig. 1.

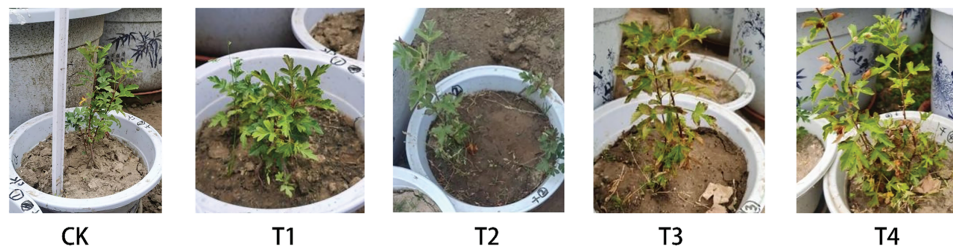


Figure 1: The phenotype under drought stress

2.2 Total RNA Extraction and Transcriptome Sequencing

Total RNA extraction was carried out on the recommended protocol of the TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China). The integrity of RNA was evaluated through a combination of 1.0% agarose gel electrophoresis (Biosharp, Hefei, China) and the Nano 6000 Assay Kit, utilizing the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Additionally, the RNA concentration and purity were assessed using the Nanodrop One (Thermo Fisher Scientific, Inc., USA).

Sequencing libraries, which were constructed with the NEBNext[®] Ultra TM RNA Library Prep Kit for Illumina[®] (Illumina, San Diego, CA, USA) and assessed with a Qubit2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent 2100 bioanalyzer (Agilent Technologies, CA, USA), were sequenced in the Illumina Novaseq platform (Illumina, USA) according to the recommended protocol.

2.3 Transcriptome Data Processing and Gene Expression Levels Analysis

The raw data was processed to guarantee the reliability and quality of subsequent analyses. Reads containing adapters, undetermined nucleotide bases (denoted as N), or exhibiting low quality—characterized by having over 50% of bases with a Qphred score ≤ 5 —were excluded. This process led to the retrieval of high-quality, clean reads. These clean reads were subsequently assembled using the Trinity software [26]. The annotations were performed based on Nr, Nt, Pfam, KOG/COG, Swiss-prot, KEGG and GO databases.

2.4 Candidate Reference Gene Screening and Primer Design

The FPKM value, which was used for estimating gene expression levels of each sequenced gene, was counted with FeatureCounts v1.5.0-p3. According to the FPKM value, candidate genes were screened based on the CV and SD value of the FPKM of each gene in different stress levels. The specified requirements are: a coefficient of variation (CV) less than or equal to 0.06, and a standard deviation (SD) not exceeding 4.0. The primer of the candidate gene was designed with Primer 3 software (<http://frodo.wi.mit.edu/primer3/>),

accessed on 18 August 2024) based on the transcriptome sequence of each candidate gene. For each candidate gene, two distinct primer pairs were designed to facilitate PCR amplification.

2.5 First Strand cDNA Synthesis and the Specificity Validation Based on qPCR

The cDNA synthesis was performed using the FastKing gDNA Dispelling RT SuperMix (TIANGEN, China), employing less than 50 ng of total RNA in a final reaction volume of 20 µL. According to the recommended thermocycling conditions, the condition is as follows: 42°C for 15 min, and then 95°C for 3 min.

All of the screened candidate genes were selected to combine PCR amplification with qPCR. The conditions of PCR amplification and qPCR were shown in our previous research about *Lycium* [27].

2.6 Data Analysis and Stability Assessment

The stability of candidate genes was assessed based on the Genorm, NormFinder, BestKeeper, and Delta Ct from qPCR.

The NormFinder (<https://www.moma.dk/software/normfinder>) (accessed on 18 August 2024, Anders D. Andersen, Aarhus University, Denmark) analyzes the stability by assessing the evaluation of stability among potential internal reference genes involves ranking them according to their stability scores. The gene exhibiting the lowest stability value is considered the most suitable reference gene [28].

The Genorm (<https://genorm.cmgg.be/>) (accessed on 18 August 2024, Ghent University, Ghent, Belgium) assesses the stability by the Average Expression Stability Value (M), the smaller M values indicate a more stable gene expression level, while higher values imply poorer stability [24].

The BestKeeper (<http://www.Gene-quantification.de/best-keeper.html>) (accessed on 18 August 2024, Biometrics, Germany) assesses the stability by analyzing the standard deviation (SD), coefficient of variation (CV), and Pearson correlation coefficient (r) of the Ct value obtained from qPCR were evaluated. Candidate reference genes demonstrated higher r values and lower SD values were considered more stable (threshold set as less than 1), smaller coefficients of variation (CV) are generally considered to reflect greater stability, whereas lower CV values suggest diminished stability [29,30].

In Delta Ct methods, the stability of candidate genes was evaluated by comparing the relative expression levels of all genes, the SD of Delta Ct value represents stability, and the smaller SD indicates more stability of the candidate gene [23]. The expression level result of each gene was drawn with Origin 2021, the Venn diagram was drawn on the website (<https://jvenn.toulouse.inrae.fr/app/example.html>, accessed on 18 August 2024).

3 Result

3.1 Sequencing Result of All Samples

A total of 96.1 G of raw base pairs were gathered from 15 samples, consisting of three control samples and 12 drought-stressed samples. Following quality control, 313,136,969 clean reads were generated. The average Q20 and Q30 values were recorded at 97.45% and 93.09%, respectively (Table A1). All raw data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the accession number PRJNA1172450.

3.2 Expression Profiles of Candidate Reference Genes

A total of 31 potential reference genes were identified through transcriptome sequencing based on FPKM values. The expression data for these genes across various developmental stages are presented in Table 1. The average FPKM values showed significant variation among the different candidate genes. The coefficient of variation (CV) ranged from 0.03 to 0.06, while the standard deviation (SD) varied between 0.17 and 3.77 (see Table 1).

Table 1: Related information about candidate reference genes

No.	Gene id	FPKM					AV	SD	CV	Swissport annotation
		CK	T1	T2	T3	T4				
1	Cluster-23533.35198	32.79	35.94	34.39	34.53	33.18	34.17	1.11	0.03	Probable WRKY transcription factor 19 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = WRKY19 PE = 1 SV = 1
2	Cluster-23533.32993	16.09	16.74	17.24	17.11	15.80	16.59	0.56	0.03	Nuclear matrix constituent protein 1 OS = <i>Daucus carota</i> subsp. <i>sativus</i> OX = 79,200 GN = NMCP1 PE = 1 SV = 2
3	Cluster-23533.47814	3.34	3.18	3.29	3.32	3.04	3.24	0.11	0.03	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8-A OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = At3g06310 PE = 3 SV = 1
4	Cluster-23533.40208	16.73	15.98	16.01	17.48	16.09	16.46	0.58	0.04	Spingoid long-chain bases kinase 1 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = LCBK1 PE = 1 SV = 1
5	Cluster-23533.42968	12.53	11.66	12.51	11.36	11.70	11.95	0.48	0.04	Probable WRKY transcription factor 20 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = WRKY20 PE = 2 SV = 1
6	Cluster-23533.45179	14.02	13.66	14.67	14.18	15.34	14.37	0.58	0.04	Protein transport protein SEC23 A OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = SEC23A PE = 2 SV = 1
7	Cluster-23533.45691	41.37	44.10	44.55	41.16	40.10	42.26	1.75	0.04	Tropolone reductase homolog At2g29340 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = At2g29340 PE = 2 SV = 1
8	Cluster-23533.43085	19.71	19.52	21.29	21.96	21.29	20.75	0.96	0.05	SUMO-activating enzyme subunit 1A OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = SAE1A PE = 1 SV = 1
9	Cluster-23533.32634	12.01	10.72	11.07	10.55	11.42	11.15	0.52	0.05	Vacuolar protein sorting-associated protein 26A OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = VPS26A PE = 2 SV = 1
10	Cluster-23533.15508	2.87	2.87	2.98	3.03	3.25	3.00	0.14	0.05	F-box/LRR-repeat protein 16 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = FBL16 PE = 2 SV = 1
11	Cluster-23533.44077	8.73	9.00	8.99	8.61	9.86	9.04	0.44	0.05	Ribosome quality control complex subunit NEMF OS = <i>Mus musculus</i> OX = 10090 GN = Nemf PE = 1 SV = 2
12	Cluster-23533.37961	51.08	52.26	57.14	57.53	52.84	54.17	2.65	0.05	Probable E3 ubiquitin-protein ligase RHC1A OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = RHC1A PE = 2 SV = 1
13	Cluster-23533.21203	5.35	5.25	5.43	5.04	5.85	5.38	0.27	0.05	KxDL motif-containing protein LO9-177 OS = <i>Oryza sativa</i> subsp. <i>indica</i> OX = 39,946 GN = LO9-177 PE = 3 SV = 1
14	Cluster-23533.38341	21.07	22.39	20.26	20.66	23.13	21.50	1.09	0.05	Clathrin interactor EPSIN 1 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = EPSIN1 PE = 1 SV = 1
15	Cluster-23533.34641	23.74	22.68	24.68	26.40	25.19	24.54	1.27	0.05	Putative vacuolar protein sorting-associated protein 24 homolog 2 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = VPS24-2 PE = 3 SV = 1
16	Cluster-23533.40778	5.82	6.71	6.35	6.73	6.49	6.42	0.33	0.05	Zinc finger CCCH domain-containing protein 38 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = At3g18640 PE = 2 SV = 1

(Continued)

Table 1 (continued)

No.	Gene id	FPKM					AV	SD	CV	Swissport annotation
		CK	T1	T2	T3	T4				
17	Cluster-23533.48337	12.40	10.99	12.03	11.35	12.60	11.87	0.61	0.05	CCR4-NOT transcription complex subunit 9 OS = <i>Bos taurus</i> OX = 9913 GN = CNOT9 PE = 2 SV = 1
18	Cluster-23533.37693	22.26	21.16	23.85	20.93	20.85	21.81	1.14	0.05	The protein DELETION OF SUV3 SUPPRESSOR 1 in NR
19	Cluster-23533.42950	30.79	31.85	32.67	28.14	29.51	30.59	1.62	0.05	Splicing factor U2af large subunit A OS = <i>Nicotiana plumbaginifolia</i> OX = 4092 GN = U2AF65A PE = 2 SV = 1
20	Cluster-23533.21950	3.19	3.73	3.57	3.36	3.41	3.45	0.18	0.05	Zinc finger protein ZOP1 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = ZOP1 PE = 1 SV = 1
21	Cluster-23533.45159	2.94	3.07	2.94	3.23	3.36	3.11	0.17	0.05	Sm-like protein LSM5 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = LSM5 PE = 1 SV = 1
22	Cluster-23533.39931	18.05	17.80	18.99	16.42	16.62	17.58	0.95	0.05	Guanine nucleotide-binding protein subunit beta OS = <i>Solanum tuberosum</i> OX = 4113 GN = GB1 PE = 2 SV = 1
23	Cluster-23533.39576	70.96	67.16	74.67	67.84	63.48	68.82	3.77	0.05	Probable serine/threonine-protein kinase DDB_G0272254 OS = <i>Dictyostelium discoideum</i> OX = 44689 GN = DDB_G0272254 PE = 3 SV = 1
24	Cluster-23533.40653	13.39	14.15	15.73	14.00	14.72	14.40	0.79	0.05	F-box protein At4g02760 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = At4g02760 PE = 4 SV = 2
25	Cluster-23533.38592	20.28	20.80	20.81	21.93	18.50	20.46	1.12	0.05	WRKY transcription factor 1 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = WRKY1 PE = 1 SV = 1
26	Cluster-23533.46015	3.70	3.98	3.88	4.31	4.24	4.02	0.22	0.06	Putative F-box/FBD/LRR-repeat protein At5g22610 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = At5g22610 PE = 4 SV = 1
27	Cluster-23533.42487	17.50	20.03	17.53	19.24	19.55	18.77	1.06	0.06	E3 ubiquitin-protein ligase BRE1-like 2 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = HUB2 PE = 1 SV = 2
28	Cluster-23533.33992	12.60	11.08	11.65	12.70	11.24	11.85	0.68	0.06	KH domain-containing protein HEN4 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = HEN4 PE = 1 SV = 1
29	Cluster-23533.42131	9.55	10.66	9.36	10.86	10.01	10.09	0.59	0.06	Serine/threonine-protein kinase 24 OS = <i>Rattus norvegicus</i> OX = 10,116 GN = Stk24 PE = 2 SV = 1
30	Cluster-23533.45050	8.32	8.57	8.68	9.69	8.30	8.71	0.51	0.06	AT-rich interactive domain-containing protein 2 OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = ARID2 PE = 1 SV = 1
31	Cluster-23533.37654	9.81	8.61	10.03	9.65	10.31	9.68	0.58	0.06	Autophagy-related protein 18a OS = <i>Arabidopsis thaliana</i> OX = 3702 GN = ATG18A PE = 1 SV = 1

Note: FPKM stands for Fragments Per Kilobase of exon model per Million mapped fragments. The terms CK, T1, T2, T3, and T4 correspond to five different treatment groups. AV denotes the average value, SD signifies the standard deviation, and CV refers to the coefficient of variation.

3.3 Amplification Specificity and Efficiency

Eighteen genes were identified that could generate distinct and clear bands, without primer dimers. Specifically amplified were selected after 1.5% agarose gel electrophoresis detection, and then used for subsequent qPCR tests (Table 2). All 18 candidate genes exhibited a distinct peak in the melting curve, demonstrating the amplification specificity of all primers (Fig. 2A–R). Most of the candidate genes showed a slight change in Ct value except for a few genes (Fig. 3A–R and Fig. A1A–1R).

Table 2: The information of candidate reference genes for qpcr

Genes	Primer		Tm	Product length	CT				Average	SD	CV	
	The forward primer (5'-3')	The reverse primer (5'-3')			CK	T1	T2	T3				T4
Cluster-23533.35198	CTGTCTGAATCTGAAATCACTTGTG	GAACAAAGTTACTGAGCTATTGGTG	58.4	275	36.17	35.44	33.96	35.76	34.45	35.16	0.92	0.03
Cluster-23533.40208	AGTGGACAGTGAACCTAAATTG	GTACCTTTGACACTTTTCCACGTA	58.0	333	26.88	25.04	26.72	27.87	28.28	26.96	1.26	0.05
Cluster-23533.42968	TACTGCTTTTGGATCATGCCGA	TTGCCAGATGAGGTTTGATGAG	58.0	282	27.38	26.07	26.15	29.01	26.71	27.06	1.21	0.04
Cluster-23533.45179	ACAAGTTTCGGCTCCCAATTT	ATTGCGCCTCACTTCCATTTC	58.0	334	31.56	30.77	30.85	32.26	32.26	31.54	0.73	0.02
Cluster-23533.37961	GTCTGGAACCGTACTTTTGAAG	CGATACAATCAGTGTGGTACAGA	58.0	305	25.85	25.05	24.82	27.12	26.03	25.77	0.91	0.04
Cluster-23533.21203	CGTTGCTATCTGAATACACTGTTG	CCTTGGTAGTAAACACCACCTCTAA	58.0	266	33.96	33.22	33.60	38.47	34.71	34.80	2.13	0.06
Cluster-23533.34641	ATTGTGAGATCTAGAAGGACAGTG	CTAGTGCTGTGTCAAGACTATCAT	58.0	259	28.02	27.37	27.71	29.40	27.63	28.03	0.80	0.03
Cluster-23533.48337	GTATAAATTGCAAGAGTGGCAGG	GAGCCCAGGATGTTAAACAATTC	58.0	319	31.28	30.08	30.11	34.00	30.94	31.28	1.61	0.05
Cluster-23533.37693	GATTGGGATGATGATGATGTCAAC	CTTCATGATTCGGAACATAACGAG	58.0	274	25.09	25.00	25.12	26.25	24.89	25.27	0.55	0.02
Cluster-23533.42950	AGATCTCCTTCTCATTTCCCGT	CGCCTTCTTACCCCAAACCATATA	58.0	316	32.29	31.89	32.93	33.59	31.93	32.53	0.73	0.02
Cluster-23533.45159	TCACTCTCTGACATACCAAGATTG	GTCTCCCTTCATTATCACC CATAT	58.0	252	27.15	27.19	26.67	28.54	25.58	27.03	1.07	0.04
Cluster-23533.39931	TCAAGACGGGCGTTTGATAG	TAGGTGGGTGTCCTCATCTG	58.0	265	27.89	27.89	28.06	30.58	26.61	28.21	1.45	0.05
Cluster-23533.39576	AGAGAGACTTTCGTGATGAATCC	CAC TTCATTTTCCCATTTTCAGGTC	58.0	306	27.00	26.33	26.74	28.53	25.86	26.89	1.01	0.04
Cluster-23533.40653	GCGGTAGTTTCCACAATGATTC	CCAAATCAACAAGAAATGGGCA	58.0	337	36.01	33.31	33.48	35.52	37.62	35.19	1.81	0.05
Cluster-23533.38592	CGACGCTTTCTCAGGTGTTA	TGCCATTCTTGTCCTTGCTT	58.0	304	36.80	36.67	37.23	38.42	34.56	36.74	1.40	0.04
Cluster-23533.33992	GGCTGATATCCGTGTTTACTCA	GATCATATCCACCAGAGCTTCC	58.0	278	36.80	36.67	37.23	38.42	37.82	37.39	0.73	0.02
Cluster-23533.45050	AATAGGTGATGTCCAAGTCTTC	TCAAAGGCTAGTTTCTATCGGAC	58.0	333	29.69	29.50	30.05	31.95	30.22	30.28	0.98	0.03
Cluster-23533.37654	TAAAGGACGTAAACGTAATAGGAGTC	TCATTCTAGGTTTATCTTTCTCCGG	58.0	295	35.74	31.24	35.57	34.85	32.07	33.90	2.09	0.06

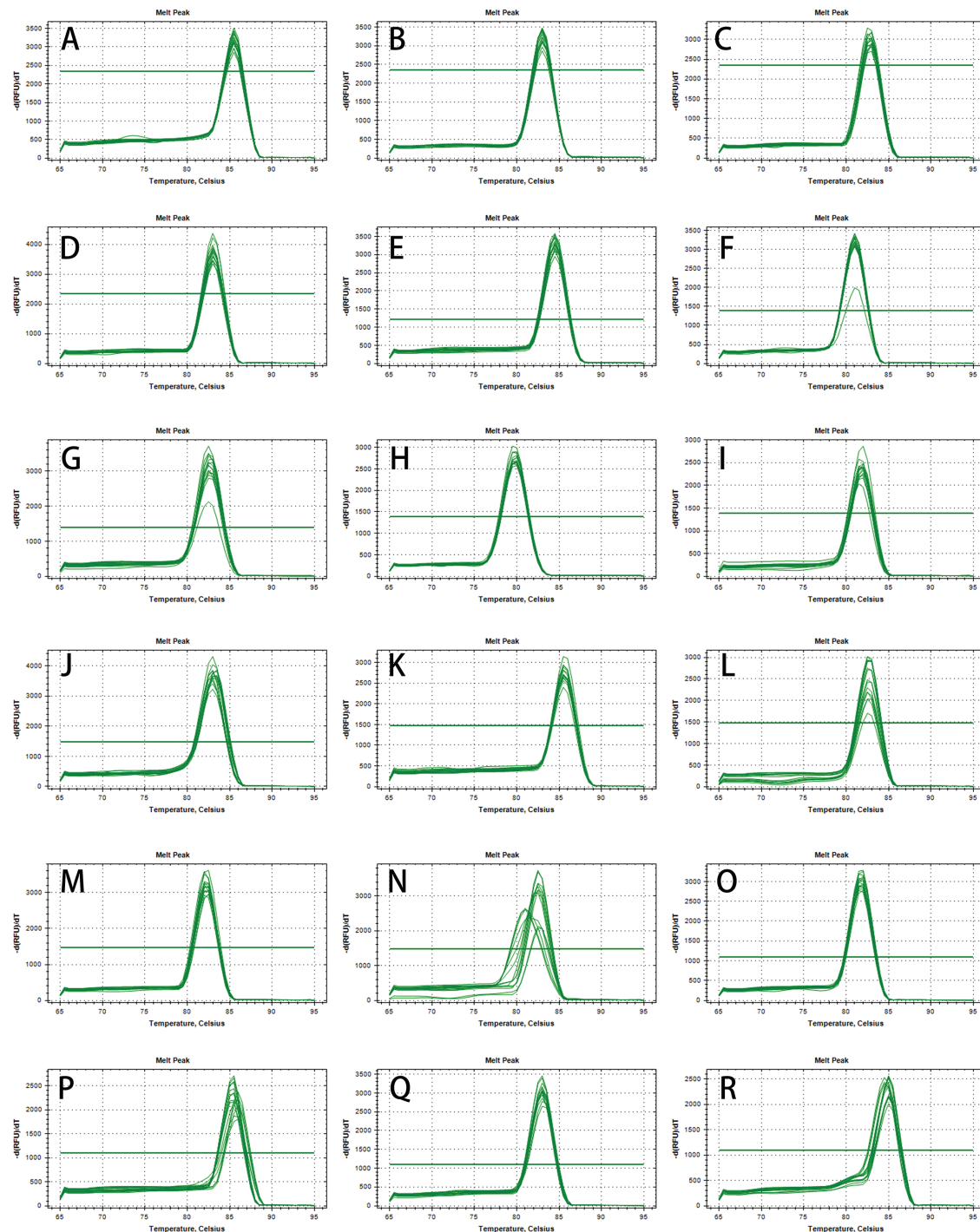


Figure 2: The melting curve analysis for each of the potential reference genes in quantitative PCR (qPCR). (A) Cluster-23533.35198. (B) Cluster-23533.40208. (C) Cluster-23533.42968. (D) Cluster-23533.45179. (E) Cluster-23533.37961. (F) Cluster-23533.21203. (G) Cluster-23533.34641. (H) Cluster-23533.48337. (I) Cluster-23533.37693 (J) Cluster-23533.42950. (K) Cluster-23533.45159. (L) Cluster-23533.39931. (M) Cluster-23533.39576. (N) Cluster-23533.40653. (O) Cluster-23533.38592. (P) Cluster-23533.33992. (Q) Cluster-23533.45050. (R) Cluster-23533.37654

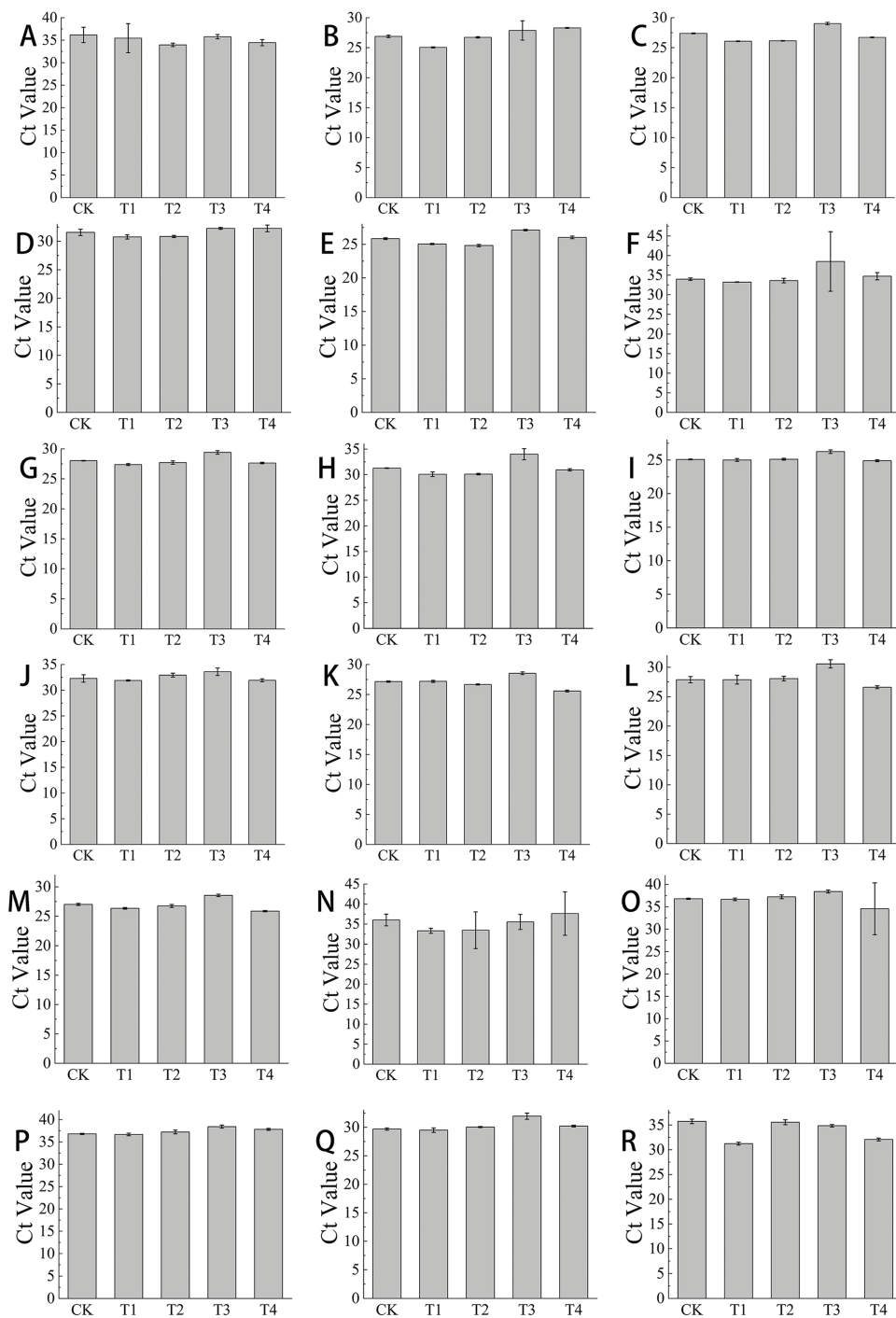


Figure 3: The Ct value of all candidate genes in qPCR (A) Cluster-23533.35198. (B) Cluster-23533.40208. (C) Cluster-23533.42968. (D) Cluster-23533.45179. (E) Cluster-23533.37961. (F) Cluster-23533.21203. (G) Cluster-23533.34641. (H) Cluster-23533.48337. (I) Cluster-23533.37693 (J) Cluster-23533.42950. (K) Cluster-23533.45159. (L) Cluster-23533.39931. (M) Cluster-23533.39576. (N) Cluster-23533.40653. (O) Cluster-23533.38592. (P) Cluster-23533.33992. (Q) Cluster-23533.45050. (R) Cluster-23533.37654

3.4 Assessment of Candidate Reference Gene Expression Stability

The stability of 18 potential reference genes was evaluated across various conditions drought stress levels was assessed using four methods based on the result of qPCR.

According to the Genorm analysis, the average expression stability (represented by numerical M value) of the 18 candidate reference genes ranged from 0.351 (Cluster-23533.34641) to 2.647 (Cluster-23533.37654). Cluster-23533.34641 and Cluster-23533.37693, Cluster-23533.39576 were identified as the most stable, as they exhibited the lowest M value among the tested genes. Conversely, Cluster-23533.40653, cluster-23533.21203 and Cluster-23533.37654, these three genes with the highest M values were identified as the most unstable (Table 3). In the result of NormFinder, the stability (represented by numerical M value) of 18 candidate genes ranged from 0.175 (Cluster-23533.34641) to 8.772 (Cluster-23533.37654), the Cluster-23533.34641 (0.175), Cluster-23533.42968 (0.178), and Cluster-23533.37961 (0.225) were the three top stable genes, The three most unstable genes identified were Cluster-23533.21203 (3.055), Cluster-23533.40653 (3.045) and Cluster-23533.37654 (8.772) (Table 3). Nearly the same as the result of Genorm and NormFinder, the result of Delta Ct analysis showed that the Cluster-23533.34641 (1.70), Cluster-23533.37961 (1.76) and Cluster-23533.42968 (1.77) were the top three stable genes, and Cluster-23533.40653 (3.61), Cluster-23533.21203 (3.68) and Cluster-23533.37654 (8.88) were the top three unstable genes (Table 3). Slightly different from the result of Genorm and NormFinder, the result of BestKeeper (represented by numerical SD and CV value) showed that Cluster-23533.37693, Cluster-23533.34641 and Cluster-23533.45179 (Table 3).

Table 3: The stability of candidate reference genes, as assessed through analyses conducted with four different software tools

Rank	Genorm		NormFinder		BestKeeper			Delta CT		
	Gene	Stability	Gene	Stability	Gene	SD	CV	Gene	SD	CV
1	Cluster-23533.34641	0.351	Cluster-23533.34641	0.175	Cluster-23533.37693	0.39	0.02	Cluster-23533.42950	1.89	0.06
2	Cluster-23533.37693	0.351	Cluster-23533.42968	0.178	Cluster-23533.34641	0.55	0.02	Cluster-23533.45179	1.88	0.06
3	Cluster-23533.39576	0.415	Cluster-23533.37961	0.225	Cluster-23533.42950	0.67	0.02	Cluster-23533.34641	1.70	0.06
4	Cluster-23533.37961	0.503	Cluster-23533.45179	0.476	Cluster-23533.45179	0.66	0.02	Cluster-23533.48337	2.01	0.06
5	Cluster-23533.42968	0.525	Cluster-23533.39576	0.531	Cluster-23533.37961	0.68	0.03	Cluster-23533.42968	1.77	0.07
6	Cluster-23533.45159	0.581	Cluster-23533.37693	0.562	Cluster-23533.45159	0.72	0.03	Cluster-23533.39576	1.77	0.07
7	Cluster-23533.42950	0.620	Cluster-23533.45159	0.719	Cluster-23533.39576	0.72	0.03	Cluster-23533.37961	1.76	0.07
8	Cluster-23533.45179	0.681	Cluster-23533.48337	0.827	Cluster-23533.33992	1.09	0.03	Cluster-23533.45159	1.89	0.07
9	Cluster-23533.48337	0.754	Cluster-23533.42950	0.859	Cluster-23533.42968	0.91	0.03	Cluster-23533.37693	1.78	0.07
10	Cluster-23533.39931	0.805	Cluster-23533.40208	1.095	Cluster-23533.38592	1.24	0.03	Cluster-23533.33992	2.65	0.07
11	Cluster-23533.40208	0.902	Cluster-23533.39931	1.101	Cluster-23533.48337	1.1	0.04	Cluster-23533.35198	2.56	0.07
12	Cluster-23533.45050	1.017	Cluster-23533.33992	1.415	Cluster-23533.35198	1.31	0.04	Cluster-23533.39931	2.06	0.07
13	Cluster-23533.35198	1.140	Cluster-23533.35198	1.681	Cluster-23533.45050	1.15	0.04	Cluster-23533.40208	2.16	0.08
14	Cluster-23533.33992	1.281	Cluster-23533.45050	1.952	Cluster-23533.40208	1.03	0.04	Cluster-23533.45050	2.50	0.08
15	Cluster-23533.38592	1.457	Cluster-23533.38592	2.373	Cluster-23533.39931	1.08	0.04	Cluster-23533.38592	3.10	0.08

(Continued)

Table 3 (continued)

Rank	Genorm		NormFinder		BestKeeper			Delta CT		
	Gene	Stability	Gene	Stability	Gene	SD	CV	Gene	SD	CV
16	Cluster-23533.40653	1.672	Cluster-23533.21203	3.045	Cluster-23533.21203	1.78	0.05	Cluster-23533.40653	3.61	0.10
17	Cluster-23533.21203	1.868	Cluster-23533.40653	3.055	Cluster-23533.40653	2.28	0.06	Cluster-23533.21203	3.68	0.11
18	Cluster-23533.37654	2.647	Cluster-23533.37654	8.772	Cluster-23533.37654	4.33	0.13	Cluster-23533.37654	8.88	0.26

Venn diagram of the most stable reference genes based on four analyzing methods showed except the Cluster-23533.40208, Cluster-23533.48337 and Cluster-23533.39931, the rest of the candidate genes are common to the results of the four analysis methods. According to their stability, Cluster-23533.34641, Cluster-23533.37693 and Cluster-23533.39576 were the top three stable genes (Fig. 4). Based on a comprehensive comparison of several analysis methods, we recommend Cluster-23533.34641 as the priority reference gene for *M. transitoria* under drought stress.

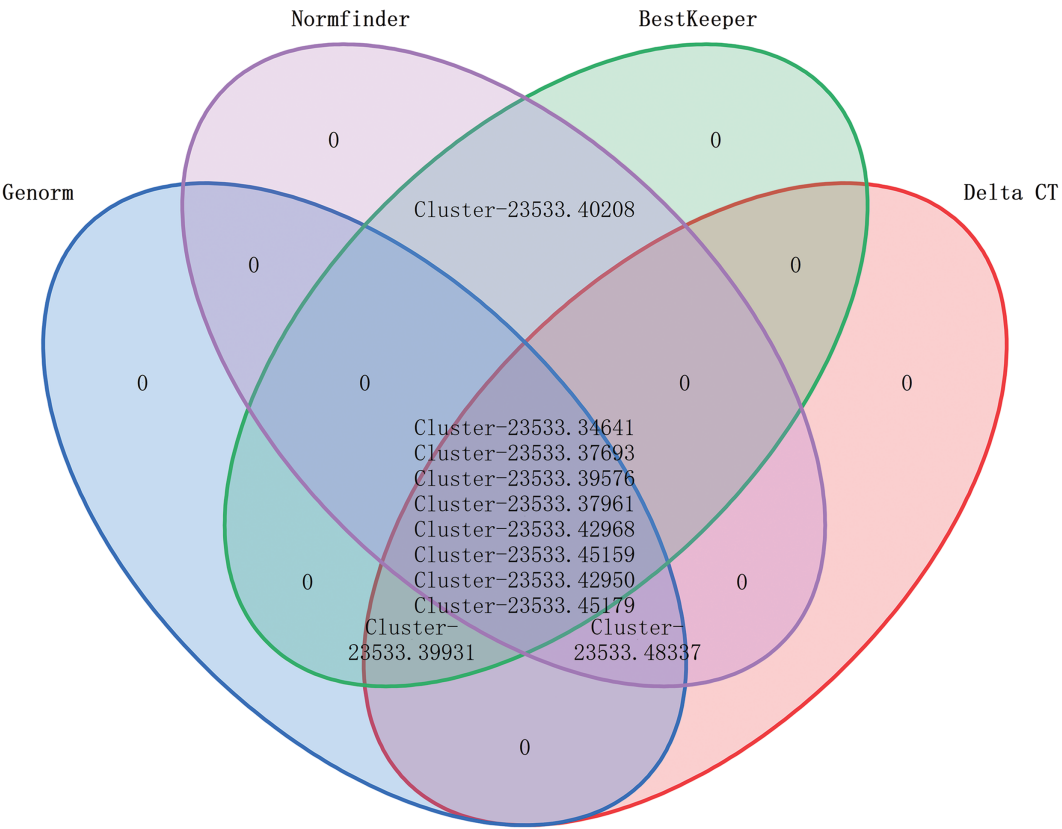


Figure 4: The Venn diagram of candidate genes

4 Discussion

With climate change, resistant plant breeding is receiving increasing attention from researchers, in particular, the functional research on resistance-related genes is the most critical for breeding. Developing and assessing the most appropriate reference gene is the key step and fundamental for gene function research.

Booming sequencing technology affords convenience for gene function research, and reference gene development and assessment also hitched a ride on technological development. Based on the Transcriptome sequencing, four new reference genes were developed and validated in drought-stressed soybeans [31]. Fifteen candidate reference genes were found through mining the publicly available transcriptional sequencing of *Solanum lycopersicum* [32].

The Cluster-23533.34641 was annotated as the putative vacuolar protein sorting-associated protein 24 (VPS) (Table 1), VPS including numerous family members, such as VPS34, VPS35, VPS38 and other members. These members are related to intracellular transport [33], sorting of vacuolar proteins [34], calcium-dependent lipid binding [35], perivacuolar compartment [36], and even related to the salt resistance of plants [37]. However, there is no report about the VPS 24 in *M. transitoria*, and its function needs to be verified.

The Cluster-23533.37693 was annotated as the protein deletion of SUV3 suppressor 1 in NR, and related to proteasome assembly, and mRNA export from the nucleus in bioprocess. The Cluster-23533.39576 was annotated as serine/threonine-protein kinase, most serine/threonine-protein kinases act as a regulator of plants when tolerance to stress, for example, SNF1-type serine/threonine protein kinase could enhance stress tolerance in *Arabidopsis* in drought, salt, and freezing stresses [38], the same result also found in rice [39]. In addition, serine threonine-protein kinase is related to the plant disease resistance gene [40,41]. The Cluster-23533.37961 was annotated as the E3 ubiquitin-protein ligase RHC1A, according to previous research it acted as a negative regulatory factor of LpbZIP40, it may be related to the high-temperature resistance mechanism of ryegrass [42]. The Cluster-23533.42968 was annotated as WRKY transcription factor 20, in some plants it is considered a conserved drought-up-regulated transcription factor [43]. Its over-expression could promote drought tolerance in *Arabidopsis thaliana* [44]. However, Cluster-23533 (Probable WRKY transcription factor 20) were stably expressed in *M. transitoria*.

According to this result, we suspected that the function of the above-mentioned candidate genes is not the same as that in other species. However, due to the lack of a reference genome, we cannot determine the functions of these genes in *M. transitoria*, so further research into gene function is needed.

In a word, the Cluster-23533.34641 was validated as the preferred reference gene for *M. transitoria* and for other related species under drought stress, and the rest reference genes could be combined and used as double internal references.

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Availability of Data and Materials: The data that support the findings of this study are openly available in the National Center for Biotechnology Information (NCBI) SRA database (Accession number PRJNA1172450).

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest to report regarding the present study.

Abbreviation

Sample	Raw Reads
qRT-PCR	quantitative Reverse Transcription PCR
UBQ	Ubiquitin extension
EF1	Elongation Factor 1
18S rRNA	18S ribosomal RNA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ACTB	Actin isoform B
ACT	Actin
UBQ-1	Ubiquitin-1
PP2A	phosphatase 2A
RPL2	Ribosomal protein L2
TUB	Tubulin
MDH	Malate dehydrogenase
THFS	formate-tetrahydrofolate ligase
RPL4	Ribosomal protein L4
TMp1	type 1 membrane protein
RPL18	Ribosomal protein L18
HIS3	Histone 3
TUA3	Tubulin α
MdH1	Histone 1

Appendix A

Table A1: Sequencing information of all samples

Sample	Raw reads	Raw bases	Clean reads	Clean bases	Error rate	Q20	Q30	GC pct
CK1	21594149	6.5	21163235	6.3	0.03	97.43	93.00	46.48
CK2	21573327	6.5	21161855	6.3	0.03	97.43	93.01	46.88
CK3	21827706	6.5	21359543	6.4	0.03	97.70	93.64	46.76
T1_1	21587576	6.5	21194088	6.4	0.03	97.52	93.22	46.77
T1_2	21533191	6.5	21028288	6.3	0.03	97.65	93.52	46.92
T1_3	20230836	6.1	19273314	5.8	0.03	97.31	92.82	46.53
T2_1	20773243	6.2	20315062	6.1	0.03	97.30	92.82	45.82
T2_2	21101759	6.3	20632463	6.2	0.03	97.41	92.99	46.32
T2_3	21842535	6.6	21320751	6.4	0.03	97.27	92.65	46.63
T3_1	21434510	6.4	21053999	6.3	0.03	97.20	92.53	46.75
T3_2	22668854	6.8	22267204	6.7	0.03	97.74	93.77	47.01
T3_3	20813870	6.2	20433872	6.1	0.03	97.27	92.66	46.74
T4_1	21048730	6.3	20529791	6.2	0.03	97.67	93.59	46.64
T4_2	21211853	6.4	20810699	6.2	0.03	97.19	92.49	46.80
T4_3	21010239	6.3	20592805	6.2	0.03	97.69	93.60	46.49

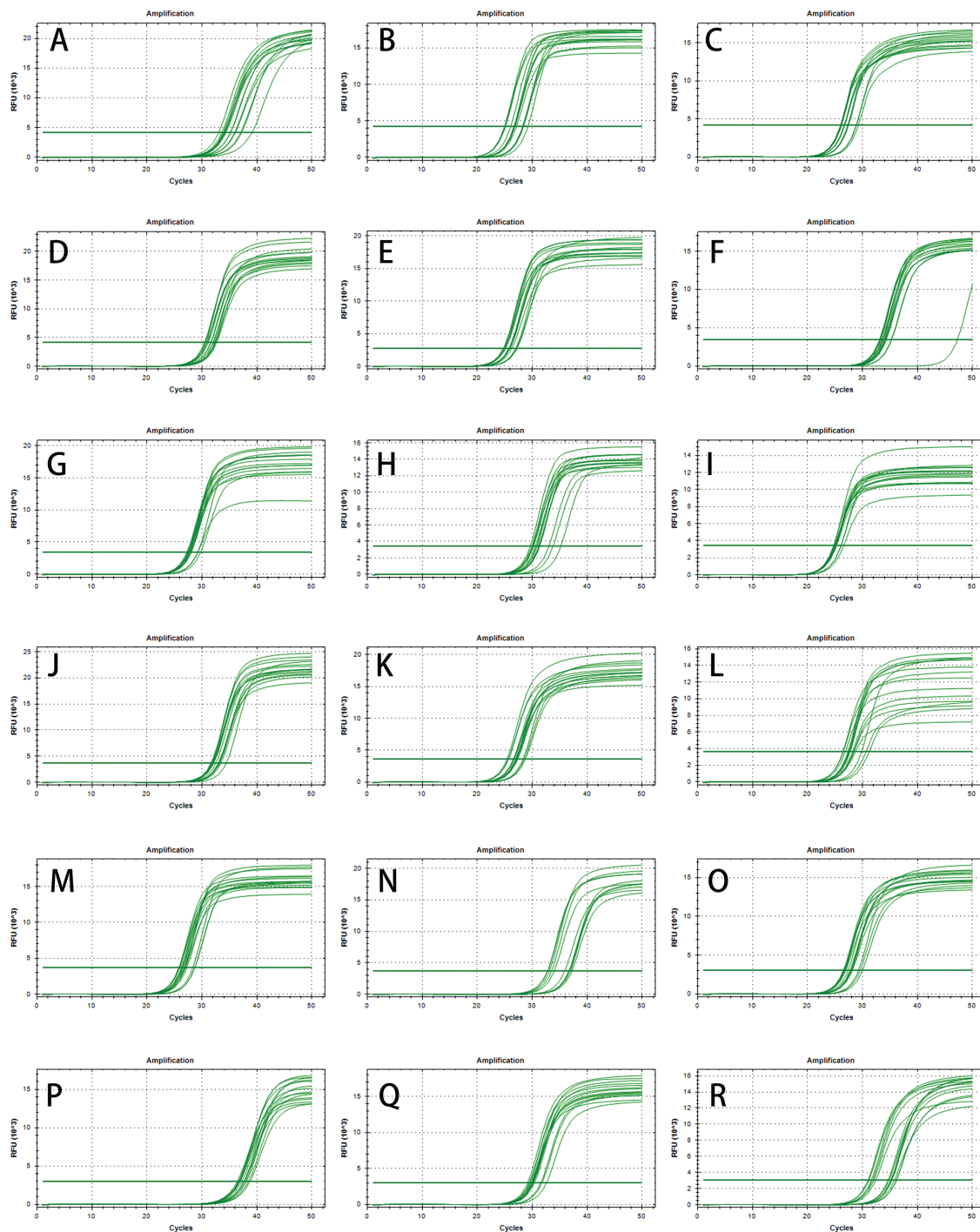


Figure A1: The amplification curve of all candidate reference gene in qPCR. (A) Cluster-23533.35198. (B) Cluster-23533.40208. (C) Cluster-23533.42968. (D) Cluster-23533.45179. (E) Cluster-23533.37961. (F) Cluster-23533.21203. (G) Cluster-23533.34641. (H) Cluster-23533.48337. (I) Cluster-23533.37693. (J) Cluster-23533.42950. (K) Cluster-23533.45159. (L) Cluster-23533.39931. (M) Cluster-23533.39576. (N) Cluster-23533.40653. (O) Cluster-23533.38592. (P) Cluster-23533.33992. (Q) Cluster-23533.45050. (R) Cluster-23533.37654. This picture is automatically generated by the machine when doing experiments, can not be modified for people, please forgive me

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