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ARTICLE

Identification of Suitable Reference Genes for qRT-PCR Normalization in *Tilia miqueliana* Maxim

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ABSTRACT

Quantitative real-time polymerase chain reaction (qRT-PCR) is a rapid and effective approach toward detecting the expression patterns of target genes. The selection of a stable reference gene under specific test condition is essential for expressing levels of target genes accurately. *Tilia miqueliana*, considered endangered, is a prominent native ornamental and honey tree in East China. No study has evaluated the optimal endogenous reference gene for qRT-PCR analysis in *T. miqueliana* systematically. In this study, fifteen commonly used reference genes were selected as candidate genes, and the stabilities of their expressions were assessed using four algorithms (GeNorm, NormFiner, BestKeeper, and DeltaCt) in nine experimental datasets. The final integrated evaluation was performed using a comprehensive analysis algorithm (RefFinder). Finally, a target MYB transcription factor gene (*TmMYB*) was used to verify the accuracy of the candidate reference genes. The results showed that PP2α was the most stable in tissue set, while *RPS13* and *SAMCD* were optimal for heat and cold stress, respectively. Under waterlogging stress, *PP2α* and *TUB* were the most stable genes in the leaves and roots, respectively. *EF1α* and *PP2α* were optimal for drying stress in leaf and root tissues. *TUB* and *ACT7* were the most stable genes in the leaf and root tissues under salt stress. This is the first systematic evaluation of candidate reference genes in *T. miqueliana*, and it will benefit future studies on expression and functional analysis of target genes in *T. miqueliana*.

KEYWORDS

Tilia miqueliana; reference genes; mRNA; qRT-PCR; normalization

1 Introduction

Quantitative real-time polymerase chain reaction (qRT-PCR) is an effective and sensitive method for quantifying target gene expression [1]. However, the accuracy of qRT-PCR is affected by the stability of the reference genes, which play a key role in reducing experimental errors among samples [2]. Commonly used internal reference genes, such as actin (*ACT7*), elongation factor-1 alpha (*EF1α*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and 18 s ribosomal ribonucleic acid (RNA) (*18 s rRNA*), are usually the housekeeping genes involved in the basic biochemical metabolism process of plants, and maintain the cytoskeleton required for plant life activities [3,4]. In-depth studies show that no ideal reference gene is stably expressed independent of cells and tissues, developmental stages, or growth

conditions [5]. Therefore, studies on the identification and evaluation of potential reference genes under different experimental conditions for several species have been conducted [6–16].

Tilia L. is a genus of the family Malvaceae, which contains 23 species of deciduous trees that are distinctly distributed in temperate areas across Asia, Europe, and North America. Most species are considered to be timber and honey resources, and are cultivated worldwide for ornamental purposes [17]. *T. miqueliana*, a unique species, is widely distributed in Jiangsu, Anhui, Zhejiang, and Jiangxi Provinces, and is currently categorized under vulnerable species in China. The deep dormancy of its seeds has resulted in poor natural regeneration [18]. Environmental factors, such as temperature, light, water, and soil, determine the survival rates of *T. miqueliana* seedlings. Despite recent reports on propagation techniques of *T. miqueliana*, the molecular mechanisms of seed dormancy and seedling adaption are crucial for the protection of *T. miqueliana* wild populations [19–21]. Identifying and clarifying the expression patterns of key genes in the aforementioned biological processes will be conducive to revealing the molecular mechanisms underlying seedling germination and adaption. However, no systematic research on the selection of candidate reference genes in *T. miqueliana* has been conducted.

In this study, 15 candidate homologous genes (Actin7 (*ACT7*), Clathrin adaptor complexes medium subunit family protein (*AP47*), plasma membrane intrinsic protein 2B/aquaporin PIP2.2 (*AQP*), tubulin alpha 3/alpha 5 chain (*TUA*), tubulin beta-1 chain (*TUB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), serine/threonine protein phosphatase 2A (*PP2α*), ubiquitin-conjugating enzyme (*UBC*), ubiquitin extension protein (*UBQ10*), histone superfamily protein (*HIS*), 18 s ribosomal RNA (*18 s rRNA*), SKP1/ASK-interacting protein (*SKIP*), ribosomal protein S13 mRNA (*RPS13*), S-adenosylmethionine decarboxylase (*SAMDC*), and elongation factor 1 alpha (*EF1α*)) were selected as candidate reference genes. In 9 different tissues and under five abiotic stress treatments, the expression stabilities of these 15 genes were validated using five algorithms: GeNorm [22], NormFinder [23], BestKeeper [24], DeltaCt [25], and RefFinder [26]. To verify the suitability of the selected reference genes in *T. miqueliana*, the relative expression levels of the target gene *TmMYB* (a homolog of MYB transcription factor gene (*MYB3*: AT1G22640) in Arabidopsis) were calculated under different experimental conditions [27]. This study provides reliable reference for gene selection in qRT-PCR normalization for future studies on gene expression in *T. miqueliana*.

2 Material and Methods

2.1 Plant Materials and Stress Treatment

All test plants were preserved in the Jiangsu Provincial Germplasm Repository of Indigenous Landscape Tree (118°49′55″E, 32°3′32″N). Nine tissues, including leaf (**LF**), root (**RT**), stem (**ST**), inflorescence bract (**IB**), flower bud (**FB**), blooming flower (**BF**), fruit (**MF**), immature seed (**US**), and mature seed (**MS**), were collected from three mature plants of *T. miqueliana* (Appendix A). For stress treatment, *T. miquelian* 1-year-old plantlets were grown at 24°C, 80% humidity, and a 16 h light/8 h dark photoperiod under LED lamps (300 μmol·m^{-2}·s^{-1}). After 15 days the plantlets began to form new leaves, they were exposed to various stress conditions including heat, cold, waterlogging, drying and salt. Tissues were collected from 63 seedlings subjected to each stress condition at 0, 1, 3 h, 6, 12, 24, and 48 h. Three different plants were harvested randomly at each sampling time and mixed for one biological replication. We repeated this sampling three times as three biological replicates. All collected samples were frozen in liquid nitrogen immediately and maintained at −80°C until RNA isolation.

For inducing heat or cold stress, the plantlets were exposed to a chamber at 40°C or 4°C and leaves were obtained using the sampling method described above. For inducing waterlogging stress, the plantlets were flooded with deionized water at a level of 2 cm above the mixture surface. For subjecting to drying and salt stress, the plantlets were placed in PEG 6000 (15%) and NaCl (52 mmol·L^{-1}) solutions. Both leaves and roots were obtained by waterlogging, drying, and salt treatments.

2.2 RNA Extraction and Complementary Deoxyribonucleic Acid Synthesis

Total RNA was isolated using the Plant RNA Extraction Kit with DNAse I (No. 0416-50 GK, Huanyueyang, Beijing, China). The integrity of total RNA was assessed using 2.0% agarose gel electrophoresis, and the RNA concentration and purity were measured with a spectrophotometer (NanoDrop 2000, Thermo Scientific, Massachusetts, USA). The cDNA was synthesized using 1 μg total RNA with the Primescript RT reagent kit (Takara, Dalian, China).

2.3 Selection of Candidate Reference Genes and Primer Design

Fifteen candidate genes, including *18S rRNA*, *ACT7*, *AQUA*, *AP47*, *EF1α*, *GAPDH*, *HIS*, *PP2α*, *RPS13*, *SAMDC*, *SKIP*, *TUA*, *TUB*, *UBC*, and *UBQ10*, were selected from the transcriptome sequences of *T. miquelina*. The closest *Arabidopsis* homologs were identified using the TAIR BLAST http://www.arabidopsis.org/Blast/index.jsp. All candidate reference genes were cloned and confirmed, and the sequences were uploaded to the NCBI database (Appendix B). Primers were designed using Oligo Primer Analysis Software Version 7 to have melting temperatures ranging between 60°C and 70°C, primer lengths between 19 and 25 bp, and amplicon lengths between 80 and 200 bp.

2.4 Quantitive Real-Time PCR Analysis

The qRT-PCR reactions were performed using the StepOne™ Real-Time PCR System (Applied Biosystems, USA) with SYBR® Premix Ex TaqTM II (Takara, Dalian, China) under the following cycling conditions: 94°C for 2 min, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, followed by melting curve analysis by heating the PCR products from 60°C to 95°C. The reaction mixture (total 20 μL) contains 1 μg cDNA, 0.6 μM of each primer, 1 × SYBR Premix. Each qRT-PCR analysis was performed in triplicate as technical repetition.

2.5 Stability Analysis of the Candidate Reference Gene

The PCR efficiency (E) and correlation coefficient (R^2) for each primer pair were evaluated using the LinRegPCR program [28]. The stability of the 15 potential reference genes was assessed using four programs: GeNorm [22], NormFinder [23], BestKeeper [24], and DeltaCt [25]. Finally, RefFinder [26] was used to integrate and rank candidate genes synthetically.

2.6 Validation of Identified Reference Genes

The top-and lowest-ranked genes were used to calculate *TmMYB* gene expression to assess the effectiveness of the reference genes. The fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [29].

3 Results

3.1 Verification of Amplication and Efficiency of the Primer

PCR products were verified by agarose gel electrophoresis and sequencing. DNA bands with the expected size in gel electrophoresis were isolated and sequencing (Appendix C). PCR amplification specificities were confirmed by melting curves. The presence of a single peak indicates that the expected amplicons were amplified (Appendix D). The correlation coefficient (R^2) values ranged between 0.981 and 0.997, and PCR amplification efficiencies ranged between 1.79 and 1.90 (Appendix B).

3.2 Expression Profile of Candidate Reference Genes

To evaluate the stability of all candidate reference genes across all 192 experimental samples, the transcript abundance of the 15 reference genes was assessed based on the mean Ct values (Fig. 1). The mean Ct values for the 15 candidate reference genes ranged from 13 to 31. Among all candidate genes, 18s rRNA ($Ct = 12.68 \pm 3.82$) had the highest expression level, whereas *TUA* ($Ct = 30.81 \pm 3.13$) was the

least expressed gene. *SKIP* (28.34 ± 1.75) was the most stable gene, while *UBC* (26.00 ± 4.42) was the most variable gene (Appendix E).

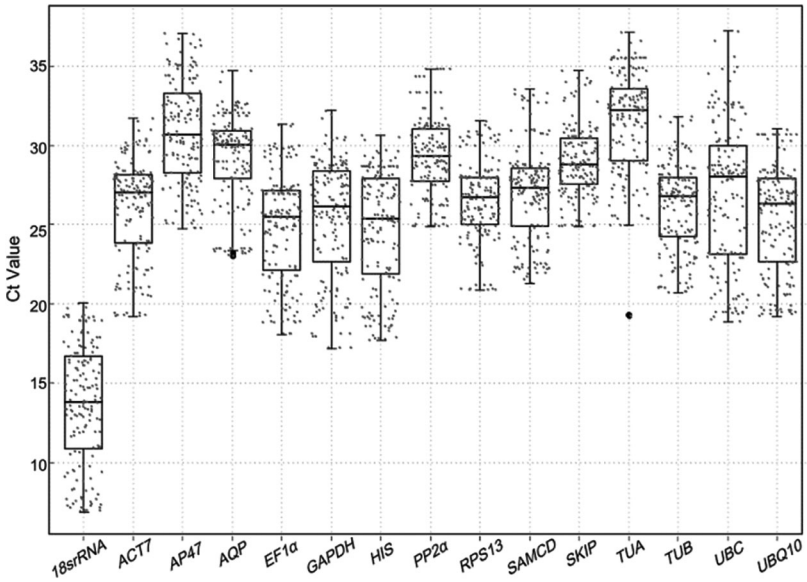


Figure 1: Box plots of the Ct values of 15 candidate genes in all 192 *T. miquelina* samples. Bold line across the box indicates the median; box indicates the 25th and 75th percentiles; whisker caps represent the maximum and minimum values; small dots represent all values in the test; and big dots represent outliers

3.3 GeNorm Analysis

According to the threshold of the M value recommended by the GeNorm program, a candidate gene could be used as a reference gene only when its M value is less than 1.5. As shown in Fig. 2 and Appendix F, a subset of genes showed expression instability with M values exceeding 1.5 (*UBQ10*, *APQ*, *RPS13*, *AP47*, *TUA*, *SKIP*, and *TUB* in tissue sets; *UBC* in leaves under drying stress; *UBQ10*, *UBC*, *AP47*, *18sRNA* and *AQP* in all samples). The two most stable genes were *HIS* and *PP2α* (M = 0.87 in nine tissues [**Tissue**]), *SAMCD* and *TUA* (M = 0.20 under heat stress [**HT**]), *EF1α* and SAMCD (M = 0.20 under cold stress [**CT**]), *PP2α* and *SKIP* (M = 0.22 in leaves under waterlogged stress [**WL**]), *ACT7* and *EF1α* (M = 0.35 in roots under waterlogged stress [**WR**]), *ACT7* and *EF1α* (M = 0.38 in leaves under drying stress [**DL**]), *EF1α* and *HIS* (M = 0.18 in roots under drying stress [**DR**]), *ACT7*, and *SAMCD* (M = 0.08 in leaves under salt stress [**SL**]), *ACT7* and *EF1α* (M = 0.17 in roots under salt stress [**SR**]), and ACT7and EF1α (M = 0.74 in all samples [**All**]).

To obtain the optimal number of reference genes in different sets, pairwise variation (V_n/V_{n+1}) was calculated using the GeNorm program (Fig. 3). Moreover, 0.15 was used as the threshold value to determine the optimal number of reference genes (n). Nevertheless, the threshold value of 0.15 should not be regarded as a rigorous standard, and higher cut-off values of V_n/V_{n+1} were found in several reports [30–32]. In tissue sets, all pairwise variations exceeded 0.15, and the minor variation was V_9/V_{10} (0.152), which demonstrated that the top nine genes were required to normalize gene expression data. For stress samples (**HT**, **CT**, **WL**, **WR**, **DR**, and **SL**), pairwise variation values of V_2/V_3 were lower than 0.15, which suggested that the combined use of the top two genes would be effective for normalizing target gene expression analysis. The V_4/V_5 values (0.120 and 0.141) were lower than 0.15 in **DL** and **SR**, which indicated that the top four genes were required for normalization. When all samples were considered together, the $V_9/_{10}$ value (0.147) was lower than 0.15, thereby illustrating the need for the top nine genes.

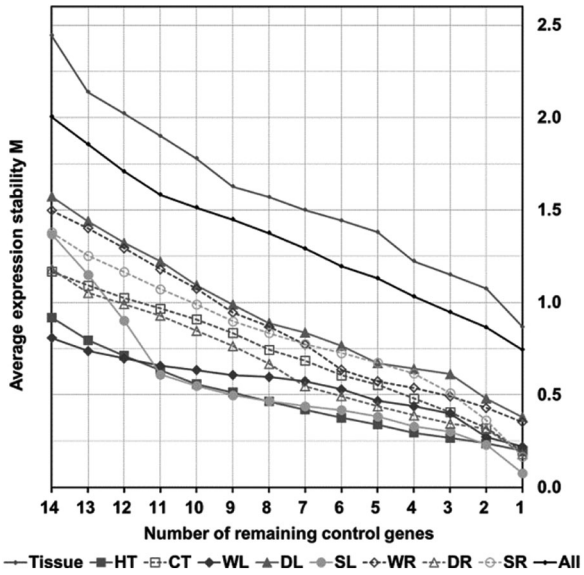
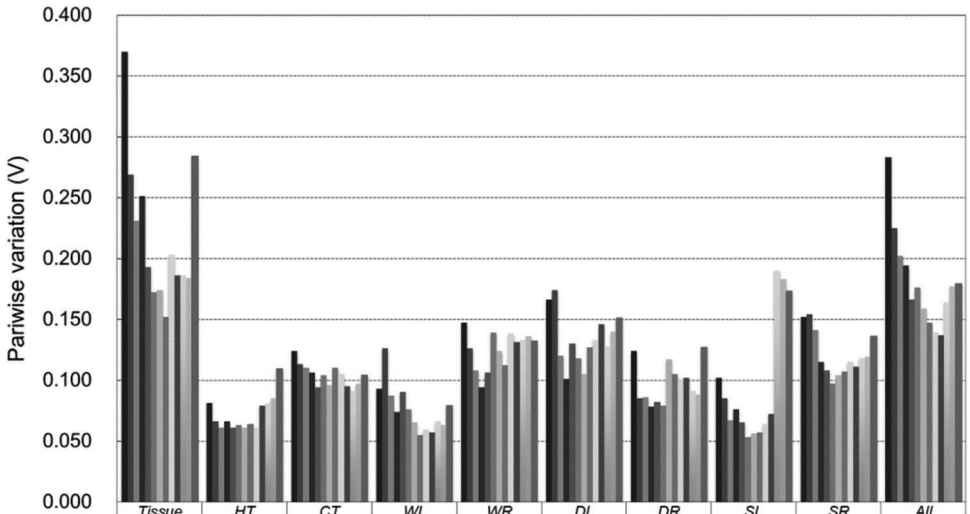


Figure 2: Average expression stability (M) of 15 candidate genes during stepwise exclusion of the least stable control gene in the different sample groups (grey small dots, **Tissue**; solid square, **HT**; hollow square, **CT**; solid diamond, **WL**; hollow diamond, **WR**; solid triangle, **DL**; hollow triangle, **DR**; solid circle, **SL**; hollow circle, **SR**; black small dots, **All**) using GeNorm analysis. The expression stability is increasing from No. 14 to No. 1. See Supplemental Table 2 for the ranking of genes according to their expression stability



	Tissue	HT	CT	WL	WR	DL	DR	SL	SR	All
V2/V3	0.370	0.081	0.124	0.093	0.147	0.166	0.124	0.102	0.152	0.283
V3/V4	0.269	0.066	0.113	0.126	0.126	0.174	0.085	0.085	0.154	0.225
V4/V5	0.231	0.061	0.110	0.087	0.108	0.120	0.086	0.067	0.141	0.202
V5/V6	0.251	0.066	0.106	0.074	0.094	0.101	0.078	0.076	0.115	0.194
V6/V7	0.193	0.061	0.094	0.090	0.106	0.130	0.082	0.065	0.108	0.166
V7/V8	0.172	0.063	0.104	0.076	0.139	0.118	0.079	0.053	0.097	0.176
V8/V9	0.174	0.061	0.096	0.065	0.124	0.105	0.117	0.056	0.104	0.159
V9/V10	0.152	0.064	0.110	0.055	0.112	0.127	0.105	0.057	0.107	0.147
V10/V11	0.203	0.061	0.105	0.059	0.138	0.133	0.101	0.064	0.115	0.139
V11/V12	0.186	0.079	0.095	0.057	0.131	0.146	0.102	0.072	0.111	0.137
V12/V13	0.186	0.081	0.091	0.066	0.133	0.128	0.091	0.190	0.118	0.164
V13/V14	0.184	0.085	0.097	0.063	0.136	0.140	0.088	0.183	0.119	0.177
V14/V15	0.284	0.109	0.104	0.079	0.132	0.151	0.127	0.173	0.136	0.179

Figure 3: Optimal number of reference genes for accurate normalization calculated by GeNorm during different tissue treatments. Pairwise variation (V_n/V_{n+1}) analysis of 15 candidate reference genes analyzed in 10 sample sunsets (**Tissue**, **HT**, **CT**, **WL**, **WR**, **DL**, **DR**, **SL**, **SR**, and **All**)

3.4 Normfinder Analysis

Normfinder calculated reference gene stability according to intra-and inter-group variations among all candidate genes (Appendix G). The more stable genes had the lower expression stability values. Accordingly, *SKIP* (0.48 **Tissue**), *SAMCD* (0.15 **HT** and 0.15 **CT**), *AP47* (0.27 **WL**), *TUB* (0.06 **WR** and 0.25 **SR**), *GAPDH* (0.11 **DL**), *PP2α* (0.08 **DR**), *HIS* (0.08 **SL**), and *ACT7* (0.06 **All**) had the highest expression stability, which showed large differences with the best genes assessed by GeNorm (Table 1).

3.5 Bestkeeper Analysis

BestKeeper analyzed reference gene stability using the values of standard deviation (SD), the coefficient of variation (CV), and correlation coefficient (r). The lower SD value of genes showed higher expression stability (Appendix H). Hence, *UBC* (1.58 **Tissue** and 1.55 **SR**), *ACT7* (0.71 **HT**), *RPS13* (0.74 **CT** and 1.70 **DL**), *SAMCD* (0.53 **WL**), *SKIP* (1.59 **WR**), *UBQ10* (0.79 **DR**) and *EF1*α (0.29 **SL**) exhibited the optimal expression stability, which was completely different from that of the first stable genes according to GeNorm and NormFinder results (Table 1). When the SD threshold was set at 1 [24], suitable reference genes could not be obtained in **Tissue**, **SR**, **DL** and **WR**.

3.6 Delta CT Analysis

The values of standard deviation (SD) were used to evaluate the expression stability of candidate genes by Delta Ct. The gene with the lowest SD value indicated the highest stable reference gene (Appendix I). Accordingly, *SKIP* (2.19 **Tissue**), *RPS13* (0.76 **HT**), *SAMCD* (0.95 **CT**), *UBQ10* (0.74 **WL**), *TUB* (1.22 **WR**, 1.04 **SL**, and 1.22 **SR**), *GAPDH* (1.28 DL), *PP2*α (0.90 **DR**), and *ACT7* (1.94 **All**) showed the highest expression stability, which was similar to that of the top stable genes calculated using NornFinder (Table 1).

3.7 RefFinder Analysis

As shown in Table 1, the most stable reference genes according to the four aforementioned algorithms were different in all experimental sets. RefFinder, a comprehensive analysis tool for expression stability of reference genes, was used to calculate the synthetic ranking of 15 potential reference genes. The top two most stable genes were identified under different experimental conditions, including **tissue** (*PP2α* and *SKIP*), **HT** (*RPS13* and *SAMCD*), **CT** (*SAMCD* and *EF1α*), **WL** (*PP2α* and *SAMCD*), **WR** (*TUB* and *TUA*), **DL** (*EF1α* and *GAPDH*), **DR** (*PP2α* and *HIS*), **SL** (*TUB* and *GAPDH*), **SR** (*TUB* and *RPS13*), and **All** (*ACT7* and *TUB*). The least stable genes in various samples calculated using the four aforementioned algorithms were basically the same, except for the **WR**, **DL**, **DR**, **SL**, and **SR** samples, where two of the least stable genes were obtained.

3.8 Validation of the Stability of Reference Genes

To detect the expression stability of the reference genes, we selected the two most stable reference genes and one unstable gene to analyze the expression patterns of *the MYB* transcription factor gene (*TmMYB*) under different tissues and experimental conditions (Fig. 4).

In **tissue**, *TmMYB* showed different expression profiles as calculated by the three references. The relative expression values of *TmMYB* normalized with *PP2*α were higher than those normalized with *SKIP* and *UBQ*. Compared with the leaf tissue, *TmMYB* was upregulated in all samples except in inflorescence bract tissue, with high values of 58, 13, and 11 in **BF**, **RT**, and **MF** normalized with *PP2*α. However, *TmMYB* expression levels normalized with SKIP in **BF** were consistent in **LT**, whereas values were downregulated in other tissues, with the lowest value (0.043) in **IB**. The relative expression patterns of *TmMYB* normalized with *UBQ10* in all samples were significantly lower than those in **LT**, and the lowest value was exhibited in the **US** (0.001).

Table 1: Expression stability ranking of the 15 candidate reference genes

M	Rank order (better-good-average)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Different tissues															
D	*SKIP*	*GAPDH*	*ACT7*	*PP2α*	*UBC*	*EF1α*	*HIS*	*TUB*	*18srRNA*	*SAMCD*	*AP47*	*RPS13*	*AQP*	*TUA*	*UBQ10*
B	*UBC*	*PP2α*	*SKIP*	*HIS*	*18srRNA*	*SAMCD*	*EF1α*	*RPS13*	*TUA*	*TUB*	*GAPDH*	*AP47*	*ACT7*	*AQP*	*UBQ10*
N	*SKIP*	*ACT7*	*GAPDH*	*EF1α*	*TUB*	*PP2α*	*UBC*	*18srRNA*	*HIS*	*AP47*	*SAMCD*	*RPS13*	*AQP*	*TUA*	*UBQ10*
G	*HIS/PP2α*		*SAMCD*	*18srRNA*	*UBC*	*GAPDH*	*ACT7*	*EF1α*	*TUB*	*SKIP*	*TUA*	*AP47*	*RPS13*	*APQ*	*UBQ10*
R	***PP2α***	***SKIP***	***HIS***	***UBC***	***GAPDH***	***ACT7***	***18srRNA***	***EF1α***	***SAMCD***	***TUB***	***RPS13***	***AP47***	***TUA***	***APQ***	***UBQ10***
Heat stress															
D	*RPS13*	*SAMCD*	*TUA*	*PP2α*	*TUB*	*EF1α*	*ACT7*	*HIS*	*SKIP*	*UBQ10*	*AQP*	*GAPDH*	*AP47*	*18srRNA*	*UBC*
B	*ACT7*	*SKIP*	*RPS13*	*TUB*	*EF1α*	*TUA*	*PP2α*	*SAMCD*	*AQP*	*HIS*	*GAPDH*	*AP47*	*UBQ10*	*18srRNA*	*UBC*
N	*RPS13*	*HIS*	*SAMCD*	*TUA*	*PP2α*	*UBQ10*	*SKIP*	*TUB*	*EF1α*	*ACT7*	*AQP*	*GAPDH*	*AP47*	*18srRNA*	*UBC*
G	*SAMCD/TUA*		*RPS13*	*TUB*	*PP2α*	*EF1α*	*ACT7*	*AQP*	*SKIP*	*UBQ10*	*HIS*	*GAPDH*	*AP47*	*18srRNA*	*UBC*
R	***RPS13***	***SAMCD***	***TUA***	***ACT7***	***TUB***	***PP2α***	***SKIP***	***EF1α***	***HIS***	***UBQ10***	***APQ***	***GAPDH***	***AP47***	***18srRNA***	***UBC***
Cold stress															
D	*SAMCD*	*EF1α*	*TUB*	*AP47*	*UBQ10*	*GAPDH*	*HIS*	*UBC*	*ACT7*	*PP2α*	*SKIP*	*AQP*	*RPS13*	*TUA*	*18srRNA*
B	*RPS13*	*TUA*	*AQP*	*SKIP*	*PP2α*	*ACT7*	*UBQ10*	*SAMCD*	*EF1α*	*TUB*	*HIS*	*UBC*	*AP47*	*GAPDH*	*18srRNA*
N	*SAMCD*	*EF1α*	*TUB*	*AP47*	*UBQ10*	*ACT7*	*PP2α*	*HIS*	*GAPDH*	*UBC*	*SKIP*	*AQP*	*RPS13*	*TUA*	*18srRNA*
G	*EF1α/SAMCD*		*AP47*	*TUB*	*GAPDH*	*HIS*	*UBC*	*UBQ10*	*ACT7*	*PP2α*	*SKIP*	*AQP*	*RPS13*	*TUA*	*18srRNA*
R	***SAMCD***	***EF1α***	***TUB***	***AP47***	***UBQ10***	***RPS13***	***ACT7***	***GAPDH***	***HIS***	***PP2α***	***APQ***	***SKIP***	***TUA***	***UBC***	***18srRNA***
Waterlogging stress in leaves															
D	*UBQ10*	*GAPDH*	*PP2α*	*TUB*	*AP47*	*SAMCD*	*RPS13*	*ACT7*	*EF1α*	*SKIP*	*TUA*	*HIS*	*AQP*	*UBC*	*18srRNA*
B	*SAMCD*	*ACT7*	*PP2α*	*SKIP*	*AQP*	*EF1α*	*UBQ10*	*GAPDH*	*RPS13*	*HIS*	*TUB*	*UBC*	*AP47*	*TUA*	*18srRNA*
N	*AP47*	*UBQ10*	*GAPDH*	*SAMCD*	*RPS13*	*PP2α*	*TUB*	*ACT7*	*EF1α*	*SKIP*	*TUA*	*HIS*	*AQP*	*UBC*	*18srRNA*
G	*PP2α/SKIP*		*TUB*	*ACT7*	*SAMCD*	*RPS13*	*EF1α*	*AP47*	*UBQ10*	*GAPDH*	*TUA*	*AQP*	*HIS*	*UBC*	*18srRNA*
R	***PP2α***	***SAMCD***	***UBQ10***	***ACT7***	***SKIP***	***GAPDH***	***AP47***	***TUB***	***RPS13***	***EF1α***	***APQ***	***TUA***	***HIS***	***UBC***	***18srRNA***
Waterlogging stress in Roots															
D	*TUB*	*TUA*	*HIS*	*ACT7*	*RPS13*	*EF1α*	*SAMCD*	*AQP*	*GAPDH*	*18srRNA*	*UBQ10*	*AP47*	*PP2α*	*UBC*	*SKIP*
B	*SKIP*	*PP2α*	*SAMCD*	*RPS13*	*TUB*	*TUA*	*ACT7*	*UBQ10*	*UBC*	*HIS*	*EF1α*	*AP47*	*AQP*	*18srRNA*	*GAPDH*
N	*TUB*	*TUA*	*HIS*	*ACT7*	*RPS13*	*EF1α*	*SAMCD*	*AQP*	*18srRNA*	*GAPDH*	*UBQ10*	*AP47*	*PP2α*	*UBC*	*SKIP*
G	*ACT7/EF1α*		*HIS*	*TUA*	*TUB*	*RPS13*	*SAMCD*	*AQP*	*18srRNA*	*GAPDH*	*UBQ10*	*AP47*	*UBC*	*PP2α*	*SKIP*
R	***TUB***	***TUA***	***ACT7***	***HIS***	***EF1α***	***RPS13***	***SAMCD***	***SKIP***	***PP2α***	***APQ***	***UBQ10***	***18srRNA***	***GAPDH***	***AP47***	***UBC***

(Continued)

Table 1 (continued)

M	Rank order (better-good-average)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Drying stress in leaves															
D	*GAPDH*	*EF1α*	*AP47*	*ACT7*	*PP2α*	*SAMCD*	*TUB*	*RPS13*	*HIS*	*AQP*	*SKIP*	*UBQ10*	*TUA*	*18srRNA*	*UBC*
B	*RPS13*	*SKIP*	*TUB*	*TUA*	*PP2α*	*EF1α*	*AP47*	*SAMCD*	*AQP*	*ACT7*	*GAPDH*	*UBQ10*	*UBC*	*HIS*	*18srRNA*
N	*GAPDH*	*AP47*	*EF1α*	*ACT7*	*PP2α*	*SAMCD*	*HIS*	*AQP*	*TUB*	*RPS13*	*SKIP*	*UBQ10*	*TUA*	*18srRNA*	*UBC*
G	*ACT7/EF1α*		*PP2α*	*RPS13*	*SKIP*	*TUB*	*AP47*	*SAMCD*	*GAPDH*	*AQP*	*HIS*	*UBQ10*	*TUA*	*18srRNA*	*UBC*
R	***EF1α***	***GAPDH***	***ACT7***	***RPS13***	***PP2α***	***AP47***	***TUB***	***SKIP***	***SAMCD***	***APQ***	***TUA***	***HIS***	***UBQ10***	***18srRNA***	***UBC***
Drying stress in Roots															
D	*PP2α*	*ACT7*	*EF1α*	*HIS*	*TUA*	*AP47*	*GAPDH*	*TUB*	*18srRNA*	*SAMCD*	*UBQ10*	*SKIP*	*AQP*	*RPS13*	*UBC*
B	*UBQ10*	*TUA*	*TUB*	*HIS*	*UBC*	*PP2α*	*SKIP*	*EF1α*	*ACT7*	*18srRNA*	*AP47*	*GAPDH*	*RPS13*	*AQP*	*SAMCD*
N	*PP2α*	*ACT7*	*TUA*	*AP47*	*HIS*	*EF1α*	*GAPDH*	*TUB*	*18srRNA*	*UBQ10*	*SKIP*	*SAMCD*	*AQP*	*RPS13*	*UBC*
G	*EF1α/HIS*		*PP2α*	*ACT7*	*AP47*	*TUA*	*GAPDH*	*TUB*	*SAMCD*	*18srRNA*	*AQP*	*UBQ10*	*SKIP*	*RPS13*	*UBC*
R	***PP2α***	***HIS***	***ACT7***	***EF1α***	***TUA***	***AP47***	***UBQ10***	***TUB***	***GAPDH***	***18srRNA***	***SKIP***	***SAMCD***	***UBC***	***APQ***	***RPS13***
Salt stress in leaves															
D	*TUB*	*AQP*	*SKIP*	*GAPDH*	*HIS*	*ACT7*	*SAMCD*	*PP2α*	*EF1α*	*RPS13*	*18srRNA*	*TUA*	*UBQ10*	*UBC*	*AP47*
B	*EF1α*	*GAPDH*	*TUB*	*HIS*	*SAMCD*	*RPS13*	*ACT7*	*AQP*	*SKIP*	*PP2α*	*18srRNA*	*TUA*	*UBQ10*	*AP47*	*UBC*
N	*HIS*	*GAPDH*	*TUB*	*AQP*	*18srRNA*	*SKIP*	*EF1α*	*ACT7*	*SAMCD*	*RPS13*	*PP2α*	*TUA*	*UBQ10*	*AP47*	*UBC*
G	*ACT7/SAMCD*		*TUB*	*SKIP*	*AQP*	*RPS13*	*EF1α*	*GAPDH*	*HIS*	*PP2α*	*18srRNA*	*TUA*	*UBQ10*	*AP47*	*UBC*
R	***TUB***	***GAPDH***	***HIS***	***APQ***	***SAMCD***	***ACT7***	***EF1α***	***SKIP***	***RPS13***	***18srRNA***	***PP2α***	***TUA***	***UBQ10***	***AP47***	***UBC***
Salt stress in roots															
D	*TUB*	*RPS13*	*ACT7*	*HIS*	*TUA*	*EF1α*	*SAMCD*	*PP2α*	*SKIP*	*18srRNA*	*AQP*	*GAPDH*	*UBQ10*	*AP47*	*UBC*
B	*UBC*	*UBQ10*	*AP47*	*18srRNA*	*AQP*	*SKIP*	*RPS13*	*TUB*	*PP2α*	*SAMCD*	*HIS*	*ACT7*	*TUA*	*GAPDH*	*EF1α*
N	*TUB*	*RPS13*	*HIS*	*ACT7*	*TUA*	*EF1α*	*18srRNA*	*SKIP*	*SAMCD*	*PP2α*	*AQP*	*GAPDH*	*UBQ10*	*AP47*	*UBC*
G	*ACT7/EF1α*		*HIS*	*TUA*	*RPS13*	*SAMCD*	*PP2α*	*TUB*	*SKIP*	*GAPDH*	*18srRNA*	*AQP*	*UBQ10*	*AP47*	*UBC*
R	***TUB***	***RPS13***	***ACT7***	***HIS***	***EF1α***	***TUA***	***18srRNA***	***SAMCD***	***UBC***	***SKIP***	***UBQ10***	***PP2α***	***APQ***	***AP47***	***GAPDH***
All samples															
D	*ACT7*	*TUB*	*GAPDH*	*HIS*	*EF1α*	*PP2α*	*SAMCD*	*TUA*	*RPS13*	*SKIP*	*18srRNA*	*AQP*	*AP47*	*UBC*	*UBQ10*
B	*SKIP*	*PP2α*	*AQP*	*TUB*	*TUA*	*RPS13*	*18srRNA*	*ACT7*	*SAMCD*	*GAPDH*	*AP47*	*EF1α*	*HIS*	*UBC*	*UBQ10*
N	*TUB*	*ACT7*	*HIS*	*EF1α*	*PP2α*	*SAMCD*	*SKIP*	*AQP*	*GAPDH*	*RPS13*	*TUA*	*18srRNA*	*AP47*	*UBC*	*UBQ10*
G	*ACT7/EF1α*		*GAPDH*	*HIS*	*SAMCD*	*TUB*	*PP2α*	*SKIP*	*RPS13*	*TUA*	*AQP*	*18srRNA*	*AP47*	*UBC*	*UBQ10*
R	***ACT7***	***TUB***	***EF1α***	***PP2α***	***HIS***	***SKIP***	***GAPDH***	***SAMCD***	***APQ***	***TUA***	***RPS13***	***18srRNA***	***AP47***	***UBC***	***UBQ10***

Note: Ranking orders evaluated by RefFinder were shown in bold. M Method, D Delt CT, B BestKeeper, N NormFinder, G GeNorm, R RefFinder.

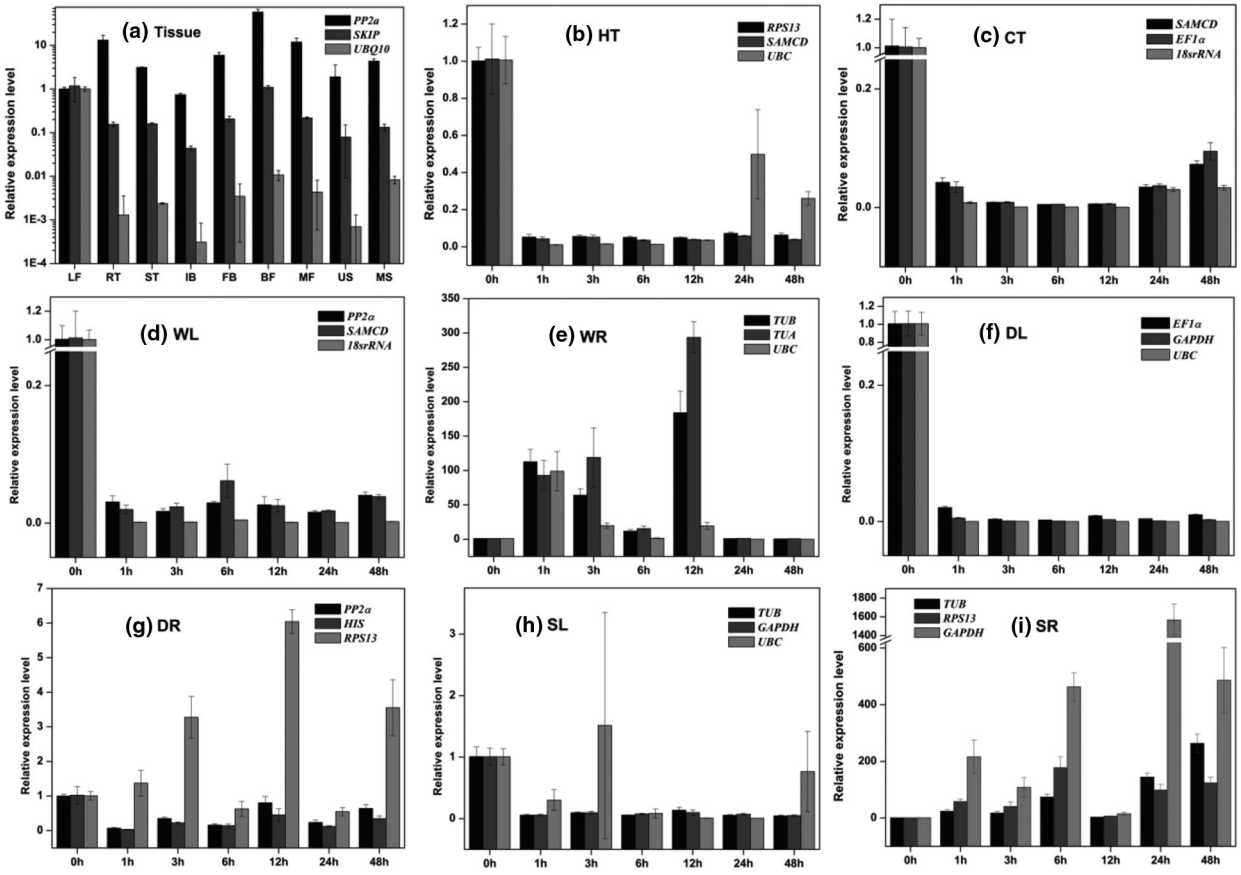


Figure 4: Relative expression levels of *TmMYB* in different experimental sets. In (a) **Tissue** set, nine tissues including **LF**, **RT**, **ST**, **IB**, **FB**, **BF**, **MF**, **US**, and **MS**. (b) **HT**, (c) **CT**, (d) **WT**, (e) **WR**, (f) **DL**, (g) **DR**, (h) **SL**, and (i) **SR**

As for stress samples, the relative expression values of *TmMYB* were reduced to extremely low levels in **HT**, **CT**, **WL**, **DL**, and **SL**. In general, compared with the reduction ranges of *TmMYB* calculated by two stable reference genes, the decline ranges normalized with the unstable genes were overvalued, and abnormally upregulated expressions were found in **HT** at 24 and 48 h and in **SL** at 3 and 48 h. Generally, the expression pattern values of *TmMYB* in **WR** increased in the first 12 h and then decreased in the subsequent time period. The expression trend of *TmMYB* peaked at 1 h or 3 h and at 12 h normalized by stable reference genes (*TUB* and *TUA*), whereas the levels of *TmMYB* were severely underestimated when analyzed using the unstable reference genes (*UBC*). Meanwhile, the expression patterns of *TmMYB* in **DR** generated by two stable reference genes (*PP2α* and *HIS*) were downregulated, but the expression level of *TmMYB* showed abnormal trends, which was upregulated when an unstable reference gene (*RPS13*) was selected for normalization. In general, the expression levels of *TmMYB* in **SR** were increased and revealed two peak values within 48 h. Nevertheless, the expression level of *TmMYB* calculated by the two optimal genes (*TUB* and *RPS13*) peaked at 6 and 48 h, whereas the maximum expression adjusted by the worst genes (*GAPDH*) occurred at 6 and 24 h with abnormally high values.

4 Discussion

Numerous studies have suggested that none of the reference genes maintain consistent expression stability under various experimental conditions. Hence, it is important to screen suitable reference genes

to accurately quantify the expression levels of target genes using qRT-PCR. There have been no relevant studies on the reference gene selection for *T. miqueliana*. Therefore, the expression stabilities of 15 candidate reference genes for *T. miqueliana* were evaluated in different tissues and in response to abiotic stresses. In the tissue sets, the *PP2α* gene was ranked first, but ranked sixth and tenth in leaf tissue under heat and cold stress, respectively. The same candidate genes have been observed to have different stabilities under different conditions [6–16]. Even under the same conditions, the most stable genes varied in diverse tissues. Under waterlogging stress, for instance, *PP2α* was ranked first in leaf tissue, whereas *TUB* was the most stable gene in root tissue. Similar results were observed for the drying and salt stresses. The results verified the need to select suitable reference genes according to the specific test conditions.

In this study, four algorithms were used to evaluate the stability of 15 candidate reference genes. In GeNorm and BestKeeper, threshold values of M (1.5) and SD (1.0) were set to evaluate the stability of reference gene. If the value is higher than the threshold, the reference gene was considered to be unstable. For the BestKeeper analysis, all candidate genes in Tissue, SR, DL and WR were inconstant according to the above-mentioned index. Nevertheless, the ranking order was still acceptable, because the expression stability is a relatively concept. Meanwhile, the threshold is not immutable, and it represents the stringency for selection of reference gene [33,34]. At the same time, discrepancies were generally observed in gene stability ranking and validation generated by the four algorithms: GeNorm, Normfinder, Bestkeeper, and Delta Ct. For instance, in all samples, *ACT7* was the most stable gene ranked by GeNorm and Delta Ct, while it was ranked fourth and eighth by Bestkeeper and NormFinder, respectively. *SKIP* was the most stable gene by Bestkeeper and was ranked in the middle or bottom position by GeNorm, NormFinder, and DeltaCt. NormFinder results showed that *TUB* was the most stable gene, while *TUB* was ranked in the middle or top position by the other three algorithms. Therefore, we recommend an evaluation of reference gene stability using more than two algorithms. However, *UBQ* was the least stable gene in all samples evaluated using the four aforementioned algorithms. Notably, in the same sample sets, three to five stable genes were obtained using the four aforementioned algorithms; however, only one or two of the most unstable genes were screened. The top stable gene was more heterogeneous than the bottom stable gene, which is attributed to differences in the calculation methods of the four algorithms [21–25]. Fortunately, integrated evaluation results can be obtained using Refinder, which is extensively acknowledged as the optimal algorithm for obtaining the final reference genes [26,35–39]. In this study, we adopted ranking results derived from the Refinder method to evaluate the stability of 15 candidate reference genes.

The *MYB* gene belongs to a large transcription factor family, which may contribute to a series of gene expressions related to plant growth, metabolism, and response to biotic and abiotic stresses [40,41]. To validate the expression stability of the reference genes, the expression patterns of the target *TmMYB* gene were detected under different conditions. Several previous reports suggested that two or more suitable reference genes would generate more reliable results [42]. Hence, we selected two stable reference genes and the least stable reference gene for normalization. In different tissues, the order of expression levels of *TmMYB* based on the three reference genes (*PP2α*, *SKIP*, and *UBQ10*) was inconsistent, hence two or more stable reference genes should be combined to calculate the relative expression levels to obtain more reliable results. Meanwhile, under stress conditions, the expression patterns of *TmMYB* showed similar trends to when the two stable reference genes were used for normalization. Hence, the use of one reference may yield reliable results, whereas two or more stable reference genes can minimize possible errors.

5 Conclusion

In this study, we systematically evaluated candidate reference genes for the normalization of gene expression data using qRT-PCR in *T. miqueliana*. In summary, the most stable reference genes were diverse in different tissues and under abiotic stress conditions. In tissue sets, *PP2α* was the most stable reference gene. Under abiotic stress, the most stable reference genes were revealed as follows: *RPS13* under heat stress, *SAMCD* under cold stress, *PP2α* and *TUB* under waterlogging stress in leaf and root tissues, *EF1α* and *PP2α* under drying stress in leaf and root tissues, and *TUB* and *ACT7* under salt stress in leaf and root tissues, respectively.

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Availability of Data and Materials: All data generated or analyzed during this study are included in this paper.

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

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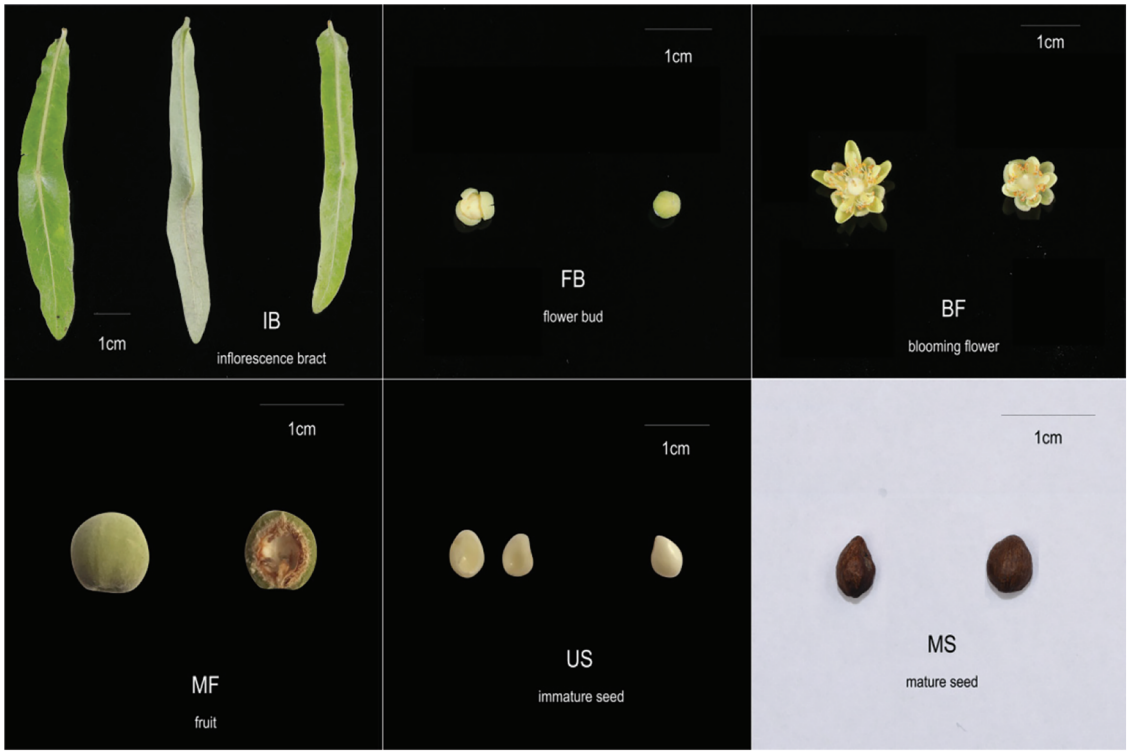
Appendix A. Phenotype of six tissues of *Tilia miqueliana* in the tissue set



Appendix B. Candidate reference genes, a target gene, Arabidopsis ortholog locus, primers and different parameters derived from quantitative real-time PCR analysis in *T. miqueliana*

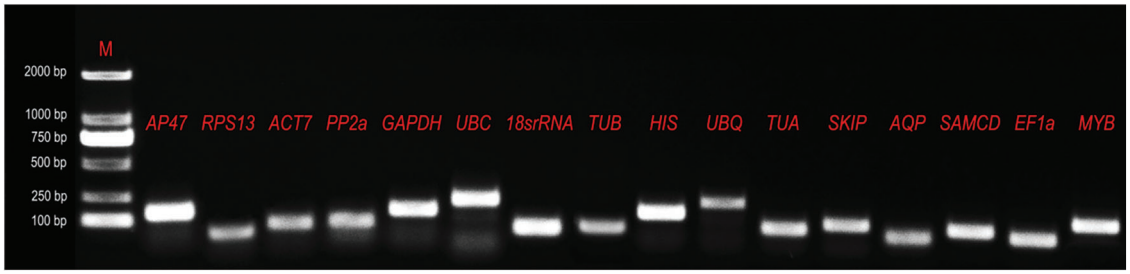
Name	GenBank	*Arabidopsis* ortholog locus	Primer pair(5'-3') Forward/Reverse	Tm (°C)	Product size(bp)	Efficiency	Correlation coefficient (R^2)
ACT7	MZ715034	AT5G09810	F: CAAGGCTAACAGAGAAAAGA R: ACTGGATAACAGAGAAAAGA	62.3/ 60.8	100 bp	1.87	0.990
AP47	MZ715035	AT5G46630	F: AACAGAGCCAACCTTGAGTGC R: ACGAGAGGCTGTAAACATTGGA	66.1/ 66.8	118 bp	1.79	0.981
AQP	MZ715036	AT2G37170	F: GAGTTCATTGCCACGCTGTTG R: GCCAAGAATGCCAACACCAC	65.3/ 65.3	111 bp	1.87	0.988
TUA	MZ715037	AT5G19780	F: CAGCCAGATCTTCACGAGCTT R: GTTCTCGCGCATTGACCATA	65.4/ 63.6	119 bp	1.89	0.993
TUB	MZ715038	AT1G75780	F: TGAACCACTTGATCTCTGCGACTA R: CAGCTTGCGGAGGTCTGAGT	66.4/ 67.2	86 bp	1.87	0.991
GAPDH	MZ715039	AT2G24270	F: GGTGTCAATGAGAAGGAATAC R: CAGTAATCGAATGGACAGTGG	59.2/ 61.4	148 bp	1.90	0.997
PP2A	MZ715040	AT3G25800	F: CCAATCCTTATTACCAGCCATCG R: CCAACACCTAACTGACTTGCC	64.1/ 64.1	108 bp	1.92	0.992

(Continued)

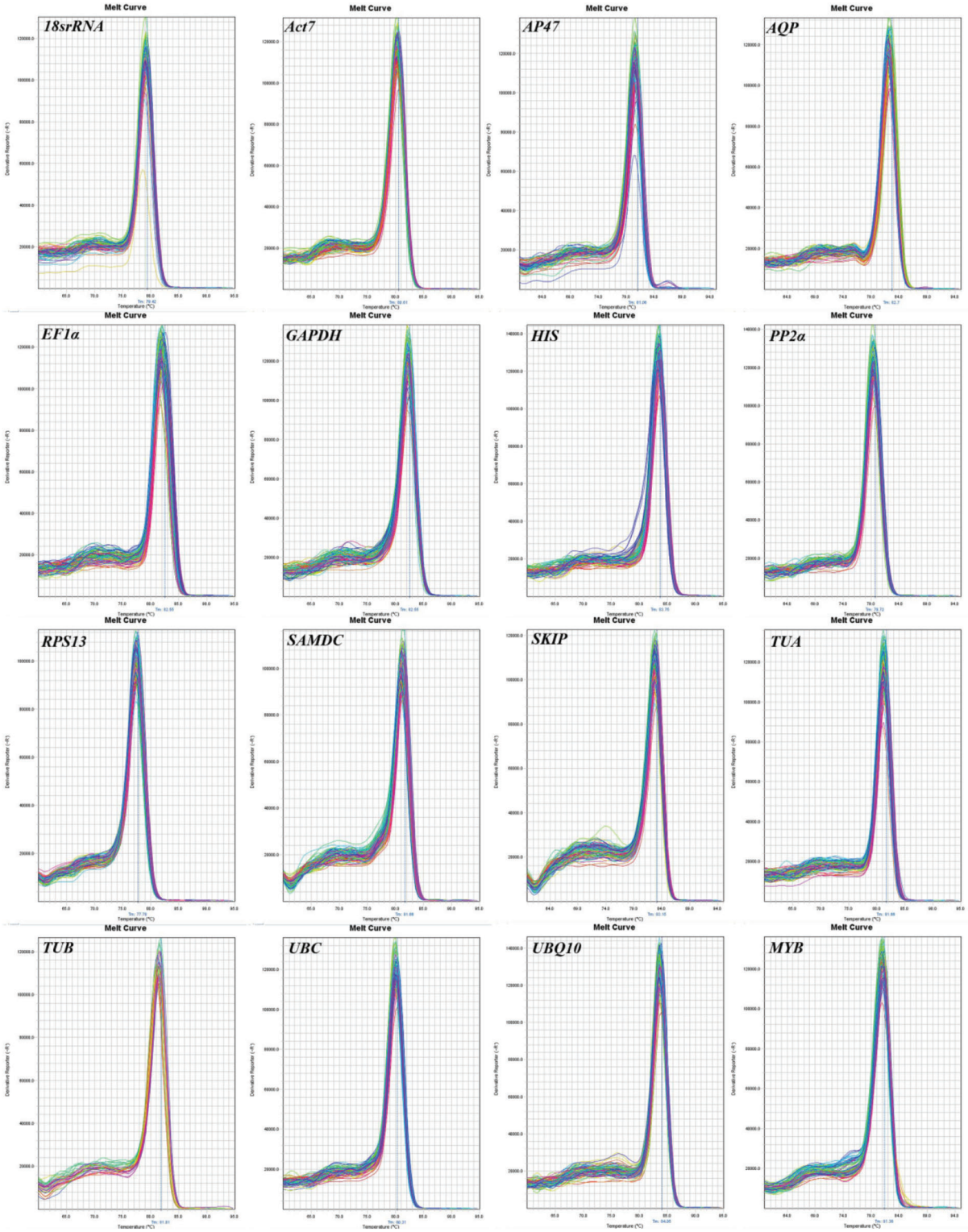
(continued)

Name	GenBank	*Arabidopsis* ortholog locus	Primer pair(5'-3') Forward/Reverse	Tm (°C)	Product size(bp)	Efficiency	Correlation coefficient (R^2)
UBC	MZ715041	AT1G64230	F: ACAAATTTCCCGGTACGTTT R: TGGATCACAAAGCAATGAC	61.2/ 59.0	208 bp	1.90	0.995
UBQ	MZ715042	AT3G52590	F: ATTTGGTTCTGCGCCTTAGAGGA R: CAGCCAGAGTACGACCGTCC	67.2/ 67.1	198 bp	1.90	0.992
HIS	MZ715043	AT4G40040	F: GTAAGTCTGCCCCAACCACC R: CAATTTCACGGACAAGCCTC	65.8/ 62.4	147 bp	1.90	0.990
18s rRNA	MZ715048	AT3G41768	F: ACCGATAGCGAACAAGTACCG R: TCCCGACAATTTCAAGCACT	65.0/ 63.1	81 bp	1.88	0.987
SKIP	MZ715044	AT1G45020	F: GAATTACGCCACCTCCAACT R: TTTCTCCAAGTTCCGTCCCAA	63.1/ 64.8	138 bp	1.84	0.982
RPS13	MZ715047	AT4G00100	F: TGAGGATTTGTACCACCTGAT R: CCTTGTCTTTCCTGTTCCTCTC	61.9/ 63.4	74 bp	1.88	0.994
SAMDC	MZ715045	AT3G02470	F: TCAGCCATATCCTCACCGTA R: TGCCATTTCTGTTTATCCAAGC	63.1/ 62.6	120 bp	1.90	0.991
EF1α	MZ715046	AT5G60390	F: GCCACACTTCCCACATTGCT R: ACCAGCATCACCGTTCTTCAA	66.4/ 65.3	113 bp	1.86	0.986
MYB	MZ976857	*AT1G22640*	F: CAACAATATCTTTCGGTGGTGCT R: CCCCAGTCTTCAATGGCTCT	64.4/ 64.8	180 bp	1.92	0.999

Appendix C. Polymerase chain reaction amplification specificity of the fifteen candidate reference genes and TmMYB, M = marker DL2000



Appendix D. Melting curves of the fifteen candidate reference genes and TmMYB



Appendix E. The statistic of qRT-PCR Ct values for all fifteen candidate reference genes in all *T. miqueliana* samples

Gene	Mean	Standard deviation (SD)	Median	Max	Min	Range
18SRNA	13.71	3.82	13.87	20.00	6.88	13.12
ACT1	26.03	3.13	27.00	31.65	19.25	12.41
AP47	30.87	3.19	30.69	37.04	24.81	12.22
AQP	29.19	2.78	30.04	34.67	23.04	11.64
EF1α	24.90	3.31	25.51	31.35	18.12	13.24
GAPDH	25.36	3.67	26.13	32.15	17.28	14.87
HIS	24.76	3.60	25.38	30.59	17.73	12.86
PP2α	29.25	2.45	29.31	34.81	24.92	9.89
RPS13	26.55	2.41	26.73	31.50	20.93	10.57
SAMCD	26.94	2.93	27.35	33.52	21.34	12.18
SKIP	28.98	2.18	28.79	34.70	24.89	9.81
TUA	31.50	3.13	32.22	37.06	19.26	17.80
TUB	26.20	2.71	26.81	31.82	20.72	11.11
UBC	26.89	4.49	28.03	37.12	18.90	18.23
UBQ10	25.45	3.42	26.30	30.96	19.28	11.68

Appendix F. Candidate reference genes ranked in order of their expression stability calculated by GeNorm under different experimental sets. The expression stability is increasing from No. 14 to No. 1. The two most stable control genes in each treatment, for example *HIS/PP2α* in tissue, cannot be ranked in order because of the required use of gene ratios for gene stability measurements. Red characters represent the average expression stability values (M) are more than 1.5. HT heat stress, CT cold Stress, WL waterlogging stress in leaves, WR waterlogging stress in roots, DL drying stress in leaves, DR drying stress in roots, SL salt stress, SR salt stress in roots

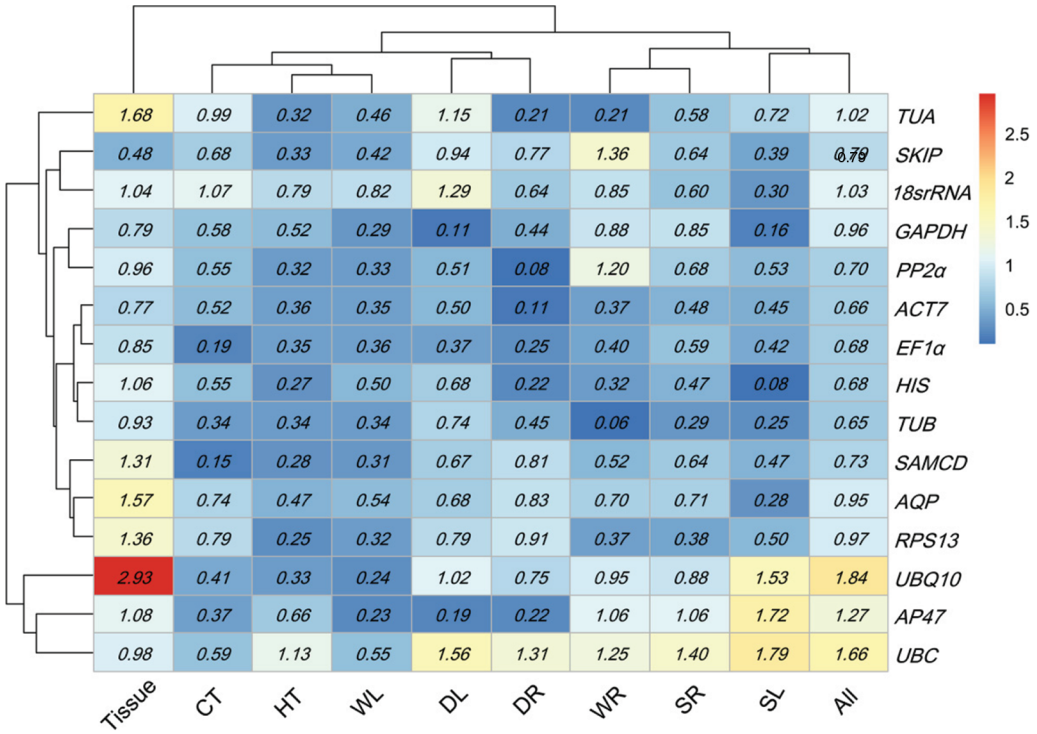
Rank	Tissue	HT	CT	WL	WR	DL	DR	SL	SR	All
14	*UBQ10*	*UBC*	*18srRNA*	*18srRNA*	*SKIP*	*UBC*	*UBC*	*UBC*	*UBC*	*UBQ10*
13	*APQ*	*18srRNA*	*TUA*	*UBC*	*PP2α*	*18srRNA*	*RPS13*	*AP47*	*AP47*	*UBC*
12	*RPS13*	*AP47*	*RPS13*	*HIS*	*UBC*	*TUA*	*SKIP*	*UBQ10*	*UBQ10*	*AP47*
11	*AP47*	*GAPDH*	*AQP*	*AQP*	*AP47*	*UBQ10*	*UBQ10*	*TUA*	*AQP*	*18srRNA*
10	*TUA*	*HIS*	*SKIP*	*TUA*	*UBQ10*	*HIS*	*AQP*	*18srRNA*	*18srRNA*	*AQP*
9	*SKIP*	*UBQ10*	*PP2α*	*GAPDH*	*GAPDH*	*AQP*	*18srRNA*	*PP2α*	*GAPDH*	*TUA*
8	*TUB*	*SKIP*	*ACT7*	*UBQ10*	*18srRNA*	*GAPDH*	*SAMCD*	*HIS*	*SKIP*	*RPS13*
7	*EF1α*	*AQP*	*UBQ10*	*AP47*	*AQP*	*SAMCD*	*TUB*	*GAPDH*	*TUB*	*SKIP*

(Continued)

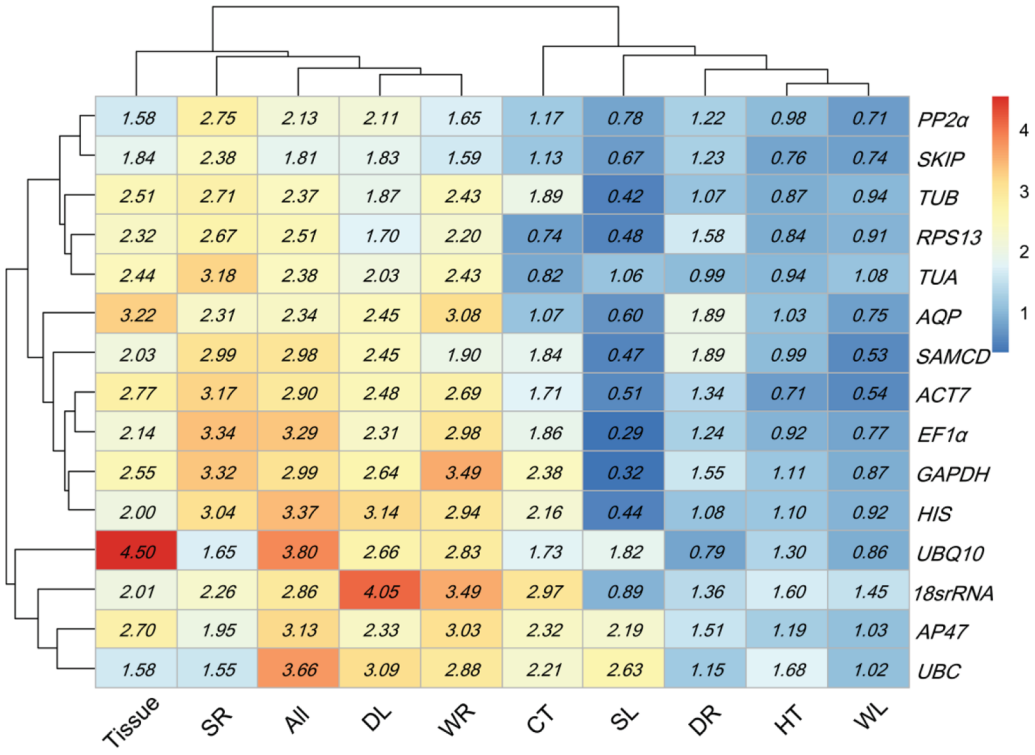
(continued)

Rank	Tissue	HT	CT	WL	WR	DL	DR	SL	SR	All
6	*ACT7*	*ACT7*	*UBC*	*EF1α*	*SAMCD*	*AP47*	*GAPDH*	*EF1α*	*PP2α*	*PP2α*
5	*GAPDH*	*EF1α*	*HIS*	*RPS13*	*RPS13*	*TUB*	*TUA*	*RPS13*	*SAMCD*	*TUB*
4	*UBC*	*PP2α*	*GAPDH*	*SAMCD*	*TUB*	*SKIP*	*AP47*	*AQP*	*RPS13*	*SAMCD*
3	*18srRNA*	*TUB*	*TUB*	*ACT7*	*TUA*	*RPS13*	*ACT7*	*SKIP*	*TUA*	*HIS*
2	*SAMCD*	*RPS13*	*AP47*	*TUB*	*HIS*	*PP2α*	*PP2α*	*TUB*	*HIS*	*GAPDH*
1	*HIS* |*PP2α*	*SAMCD* |*TUA*	*EF1α* |*SAMCD*	*PP2α* |*SKIP*	*ACT7* |*EF1α*	*ACT7* |*EF1α*	*EF1α* |*HIS*	*ACT7* |*SAMCD*	*ACT7* |*EF1α*	*ACT7* |*EF1α*

Appendix G. Expression stability for 15 candidate genes calculated via NormFinder. HT heat stress, CT cold stress, WL waterlogging stress in leaves, WR waterlogging stress in roots, DL drying stress in leaves, DR drying stress in root, SL salt stress in leaves, SR salt stress in roots, All all samples. Low to high expression stability is represented over a spectrum from red to blue, respectively



Appendix H. Expression stability for 15 candidate genes calculated via BestKeeper. HT heat stress, CT cold stress, WL waterlogging stress in leaves, WR waterlogging stress in roots, DL drying stress in leaves, DR drying stress in root, SL salt stress in leaves, SR salt stress in roots, All all samples. Low to high expression stability is represented over a spectrum from red to blue, respectively



Appendix I. Expression stability for 15candidate genes calculated via DeltaCt. HT heat stress, CT cold stress, WL waterlogging stress in leaves, WR waterlogging stress in roots, DL drying stress in leaves, DR drying stress in root, SL salt stress in leaves, SR salt stress in roots, All all samples. Low to high expression stability is represented over a spectrum from red to blue, respectively