Reference Gene Selection for miRNA qRT-PCR Analysis in Lily

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Research

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Abstract

Background: The selection of reliable reference genes is a critical element for obtaining accurate gene expression data to assess quantitative real-time polymerase chain reaction (qRT-PCR) performance. It is critical to use suitable reference genes in miRNA qRT-PCR because of short amplification products and large differences in the expression levels of target miRNAs involved in some biological processes. However, in lily, which exhibits a large complex genome but lacks a reference, the available miRNA reference genes for use in qRT-PCR under various treatment conditions are limited, and their reliability has rarely been systematically evaluated.

Results: In this study, 8 candidate reference genes, including three classic housekeeping genes and five potential miRNAs from the miRNA library of *L.* × *formolongi*, were selected and assessed for expression stability utilizing the BestKeeper, geNorm and Normfinder tools, together with the Delta Ct method, across a diverse set of biotic and abiotic experimental conditions (developmental stages, tissues, heat stress and pathogen defence) to determine the best reference gene(s) for *L.* × *formolongi* and *L. regale*. The final ranking was reordered by using RankAggreg, and the results showed that the novel miRNA PC-3p-67_108977 and the conserved miRNAs miR399a, miR399a and U6 were the most stable genes for *L.* × *formolongi* and *L. regale*, respectively, under all tested experimental conditions. Additionally, PC-3p-67_108977 and U6 were the most suitable genes for qRT-PCR studies in lily.

Conclusions: This study provides a comprehensive evaluation of the reliability of reference genes for miRNA studies on development and biotic and abiotic stress responses in different lilies. These results will be beneficial for miRNA identification and functional studies of lilies in the future.

Introduction

Lilium (Liliaceae family) includes important crop species with great economic and ornamental importance [1]. Molecular studies of lily focused on flowering time control, disease resistance and vegetative propagation have been conducted to improve agronomical traits [1, 2, 3, 4]. microRNAs (miRNAs) are endogenously expressed RNAs that have been proven to present diverse functions in biological processes including developmental progression and biotic and abiotic stress responses in both dicotyledonous and monocotyledonous plants [5, 6, 7]. Recent studies have confirmed that miRNAs play pivotal roles in the modulation of multiple biological processes in lily [6, 7, 8]. The exploration of miRNA function is required to understand the complex modulation mechanisms of biogenetic processes in lily.

Among the biochemical approaches for investigating miRNA functions, quantitative real-time polymerase chain reaction (qRT-PCR) is one of the preferred methods for monitoring and quantifying target expression levels because of its advantages in terms of sensitivity, specificity, reproducibility and informativeness [9, 10]. The proper selection of reference genes is a crucial prerequisite for the normalization of the expression levels of genes of interest [11, 12]. A suitable reference gene used as an internal control should maintain a constant expression level among different samples and different experimental systems [13, 14]. However, there are no reference genes that are universally suitable for diverse species or particular circumstances [15, 16, 17].

In plants, a set of traditional reference genes (including snRNA U6, rRNA 5S, 5.8S, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and 18S) that ubiquitously participate in basic cellular processes and are uniformly expressed in all cells are frequently used in miRNA expression analysis [18, 19, 20]. However, these genes have shown unacceptable variations in expression in some experiments [21, 22]. Recently, several conserved miRNAs, such as miR156b, miR160, and miR168, and a few novel miRNAs have been employed as reference genes for normalizing and accurately quantifying miRNA expression in several plant species. These genes are more stable than classic protein-coding genes in experiments conducted under distinct conditions, such as drought stress, osmotic stress, and somatic embryogenesis [19, 20, 23, 24]. However, the utilization of these plant miRNAs is also restricted to specific conditions because of their spatiotemporal and tissue-specific expression profiles and high expression in particular developmental stages [25, 26]. For instance, miR156 and miR160 have been confirmed to play important roles in developmental phase changes and auxin signalling responses, respectively, and exhibit conserved expression patterns during plant growth [27, 28, 29]. In addition, considering the relatively short sequences of PCR products, complex regulatory mechanisms of some biological processes and huge genome size of lily, it is critical to mine new reference genes and select reliable genes for miRNA qRT-PCR studies in this genus. However, the suitable reference genes available for use in a wide range of biological studies are limited in lily, for which no reference genome is available. Additionally, a systematic evaluation of the reliability of candidate reference genes for normalizing the relative expression levels of miRNAs in qRT-PCR studies of multiple biological processes, especially developmental transitions and abiotic and biotic stress responses, is still lacking in lily.

High-throughput sequencing technology provides rich miRNA sequence resources and abundance data for samples via the construction of small RNA libraries [30]. The *L.* × *formolongi* cultivar and the wild species *L. regale* are two particularly useful lily materials for flowering and disease-resistance studies, respectively. The former differs from most other lilies in that it can flower within one year after sowing [31]. In contrast, seedlings of *L. regale* require at least 2 years to flower, but these plants present high resistance to fungal pathogens [32]. In this study, 3 classic housekeeping genes (U6, 5S and 5.8S) and 5 miRNAs (3 conserved miRNAs and 2 novel miRNAs) identified from high-throughput data for *L.* × *formolongi* were selected to evaluate their expression stability under different experimental conditions and in various biological samples of *L.* × *formolongi* and *L. regale*. This study is the first to provide comprehensive insight into the behaviour of reference genes for use in qRT-PCR assays in two lilies under various conditions (developmental stages, tissues, biotic stress and abiotic stress) on the basis of different methods, including geNorm, BestKeeper, NormFinder, Delta Ct and RankAggreg analyses. The results could help to select suitable reference genes with application ranges that are as wide as possible and produce more accurate miRNA qRT-PCR results to better understand the molecular mechanisms mediated by miRNAs in lily.

Materials And Methods

Plant materials and stress treatments

Seeds of *L.* × *formolongi* ('Razan No.2') and *L. regale* were stratified at 4°C for approximately one month and sown in a greenhouse. According to a previous study in our laboratory, seedlings of lily must progress through a vegetative development stage (rosette stage) to reach the adult stage, in which bolting is followed by reproductive development [2]. The fresh leaves of two lilies were obtained from three individual plants at the rosette leaf stage, bolting stage and visible bud stage. All samples were immediately frozen in liquid nitrogen and stored at -80°C.

Different tissues, including fresh leaves, middle stems, bulb scales from the middle of the bulb, roots and young seeds, were also harvested from single plants in the adult stage after flowering with three biological replicates in the two lilies, followed by freezing and storage.

To investigate the stability of the selected candidate reference genes under abiotic conditions, seedlings of lilies were transplanted to pots in chambers at 5 weeks after sowing and grown under a 16 h light (32°C)/ 8 h dark (16°C) cycle with 70% humidity. Fresh leaves of the 2 kinds of lilies were collected at 0 weeks, 12 weeks and 22 weeks after heat treatment.

For biotic stress treatment, leaves were detached from the middle of the stems of *L.* × *formolongi* and *L. regale* at the flower bud stage. After cleaning with distilled water, these leaves were treated with 5×10^4 conidia•mL^{-1} of *Botrytis elliptica* via surface spraying [33] and placed in trays covered with preservative film under 90-100% humidity. The leaves were collected at 0, 6, 12, and 24 hours post inoculation (hpi) and prepared for miRNA extraction.

Small RNA extraction and cDNA synthesis

miRNA was extracted using miRcute miRNA Isolation kits (Tiangen Bio, Beijing, China) according to the manufacturer's instructions. The enzymatic addition of a poly (A) tail was performed using an *E. coli* Poly (A) Polymerase kit (Invitrogen, USA) according to the manufacturer's instructions after measuring the quality and concentration of non-poly (A) RNAs. Then, cDNA was obtained using a Quant Script RT Kit (Tiangen Bio, Beijing, China) and prepared for further qRT-PCR analysis.

Selection of candidate reference genes

Three classic housekeeping genes, 5S, 5.8S and U6, were selected, and their sequences were obtained from previous studies. Many miRNAs were identified in small RNA libraries generated in our laboratory during the vegetative development and initial flowering for *L.* × *formolongi*, and extensive sequencing data provided additional potential reference genes. Expression levels and differences in deep-sequencing counts in the form of copy numbers and p-values were also analysed in the libraries [34]. Therefore, we selected mature miRNAs that displayed consistently high expression with low variance as candidate reference genes. miRNAs with copy numbers greater than the average (total copies/ (sample numbers × total miRNAs)) in random samples were defined as showing high expression levels. In addition to conserved miRNAs, several novel miRNAs were also identified, and the secondary structures of their precursors were predicted using RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The secondary structures had to satisfy the criteria for the annotation of miRNAs reported previously [5, 35].

Primer design and qRT-PCR analysis

Specific miRNA primers were designed using the Primer-BLAST tool of NCBI (http://www.ncbi.nlm.nih.gov/tools/prime r-blast/) based on the sequences of the mature miRNAs and their precursors in the small RNA libraries according to parameters reported in a previous study [36]. The details of the primers are shown in Table 1.

qRT-PCR was carried out by using THUNDERBIRD SYBR qPCR Mix Without Rox (Toyobo, Shanghai, China) on a Bio-Rad CFX96 system (CFX96 Touch, BIO-RAD, USA). Each reaction volume of 20 μL contained 10 μL of THUNDERBIRD SYBR qPCR Mix, 4.0 μL of each diluted (1:9) primer (10 μM), and 2.0 μL of the diluted (1:9) cDNA template. A melting curve was generated for each primer pair over a temperature range of 50-59.4°C to inspect the single specific amplification products.

Standard curve analysis was conducted to determine the corresponding correlation coefficients (R^2) and the efficiency of amplification (E). The cycling program for product amplification was as follows: 95°C for 30 s (hot-start activation), followed by 40 cycles of 95°C for 10 s (denaturation) and 50-59.4°C/ 72°C for 20 s (annealing/ extension). Each reaction was performed in triplicate.

Stability assessment of candidate reference genes

The stability of candidate reference genes was examined utilizing three Excel-based algorithms tools, geNorm [13], BestKeeper [37] and NormFinder [38]. Based on Cq values representing the expression levels of each candidate, comparisons between the expression stability values (*M*) generated by these tools were conducted according to the underlying principles and calculations described in the manual. In addition, the simple delta Ct approach was utilized to compare 'pairs of genes' in samples to evaluate the expression stability of candidate reference genes [13, 39]. To determine the best reference genes, the final ranking of the set of candidates in these programs was integrated for weighted rank aggregation analysis employing the R package RankAggreg [40].

Results

Identification of candidate reference genes from miRNA libraries

In addition to 3 classic housekeeping genes, 5 potential miRNAs were derived from the high-throughput sequencing dataset. These miRNAs, with consistent (p-value > 0.05) high expression levels, were screened for further assessment. Thus, 5 potential miRNAs, including 3 conserved miRNAs (miR399a, miR2916, miR5083) and 2 novel miRNAs (PC-3p-67_108977, PC-3p-3100_2399) that simultaneously satisfied the common criteria for miRNAs, were identified in the small RNA libraries of *L.* × *formolongi* (Table 1). Hair-pin structure predictions for precursors of PC-3p-67_108977 and PC-3p-3100_2399 were produced and are shown in Fig. 1. The free energies of the thermodynamic ensemble of PC-3p-67_108977 and PC-3p-3100_2399 precursors were − 38.75 and − 58.40 kcal/mol, respectively, their minimum folding free energy indexes (MFEIs) were 1.1 and 1, and their lengths were 101 and 119 nt.

Amplification with specific primers was performed by standard PCR to target 8 candidate reference genes in lilies. Single bands of the expected lengths were produce (Additional file 1: Fig. S1). Amplification efficiencies ranging from 90.5 to 107.8 were achieved. The qRT-PCR amplification results were further analysed.

Table 1
Primer sequences and amplifications characteristics of candidate reference genes in lily

Gene	Primers	Tm(°C)	PCR efficiency	Regression coefficient(R^2)	source
5.8S	F: AAATGCCCGACATTAAGCTG	56.3	96	0.99	Jeyaraj et al., 2017[35]
	R: TTGCAATTTCTCACCATCCA				
5S	F: GTTGCAGGGTGCGATCATA	56.3	107.1	0.989	Jeyaraj et al., 2017[35]
	R: AGGGGGTCACCCATCCTAGT				
U6	F: GATAAAATTGGAACGATACAG	56.3	104.1	0.896	Wei et al., 2018[41]
	R: ATTTGGACCATTTCTCGATTT				
miR399a	F: ATGCGTGCCAAAGGAGAATTGCCCTG	59.4	90.5	0.948	This study
miR2916	F: CCATAAACGATGCCGACCAGGGAT	59.4	106.7	0.91	This study
miR5083	F: ATACCAATGGATCCTTCTGAGCCCTC	52	95.3	1.00	This study
PC-3p-3100_2399	F: CATTCCTATGTCGCTCAATCCAATAGATTC	52	107.8	0.965	This study
PC-3p-67_108977	F: TATCTTCACTGCCACCATCCGCCTG	50	104.8	0.949	This study
Universal Reverse	R: GCGAGCACAGAATTAATACGACTCAC	-	-	-	This study

Cq values of candidate reference genes

The Cq (RT-qPCR-derived quantification) values of 5.8S, 5S, U6, miR399, miR2916, miR5083, PC-3p-67_108977 and PC-3p-3100_2399 across all experiments conducted in *L.* × *formolongi* and *L. regale* were obtained through qRT-PCR. The mean Cq values varied from 18.60 (5S in *L. regale*) to 28.39 (miR399a in *L.* × *formolongi*) and showed large discrepancies between the two lilies (Fig. 2). The highest Cq values, representing the lowest expression levels, were 38.60 for miR399a in *L.* × *formolongi* and 37.3 for U6 in *L. regale*. Additionally, 5.8S and 5S exhibited the highest expression levels with the lowest Cq values in *L.* × *formolongi* (16.9 for 5.8S) and *L. regale* (11.87 for 5S). The Cq values of these candidate reference genes varied among all samples. Therefore, the Cq values of the candidate reference genes were calculated and processed for further evaluation to identify the most stable gene(s) under the experimental conditions.

Stability analysis of candidate reference genes in lily

geNorm analysis

The average expression stability (*M*) provided by the geNorm program represents the stability of the tested candidate genes. The rankings among the candidate genes in different developmental stages, tissues, and biotic and abiotic stress conditions are depicted in Fig. 3.

The candidate PC-3p-3100_2399 and miR2916, with the lowest *M* values, were the most stable genes for different developmental stages, followed by miR399a and the classic reference gene U6 in *L.* × *formolongi* and *L. regale*, respectively. Among different tissues, PC-3p-3100_2399 was the optimal normalization gene in *L.* × *formolongi*. Under different stress conditions, geNorm ranked PC-3p-67_108977 as the best reference gene in *L.* × *formolongi*. PC-3p-67_108977 was also the most highly ranked gene among different tissues in *L. regale*. Under heat stress and *B. elliptica* treatment, miR399a and miR5083, respectively, were identified as the most suitable reference genes in *L. regale*. When all the sets of samples from various conditions were analysed, U6 and miR5083 were identified as the most stable reference genes in *L.* × *formolongi* and *L. regale*, respectively. Among all samples, PC-3p-67_108977 was the ranked as the most suitable reference gene for miRNA qRT-PCR analysis in lily (Table 6).

In addition to providing rankings, geNorm estimates the stability of candidates via stepwise exclusion. The average pairwise variation (*V*) scores for the genes in relation to other candidates were obtained, followed by the stepwise exclusion of the most stable candidates. Thus, the optimal number of reference genes

for ensuring accurate normalization results was obtained using this algorithm. Nevertheless, the pairwise variation scores calculated by the program in this study were greater than 0.15, which was considered the recommended cut-off value [13] (Additional file 2: Fig. S2).

NormFinder analysis

The expression variation of overall candidate genes and intergroup samples was estimated. The stability values of single candidates were generated from the NormFinder algorithm (Table 2). The results of the NormFinder analysis were similar to those generated by geNorm in most cases, especially in *L.* × *formolongi*. The program ranked miR399a and U6 as the most stable candidates expressed during development in *L.* × *formolongi* and *L. regale* with minimum stability values of 0.920 and 0.311, respectively. Across different tissues in the two kinds of lilies, the most stable reference genes were PC-3p-3100_2399 and 5.8S in *L.* × *formolongi* and *L. regale*, respectively. The stability values of the candidates in *L. regale* ranged from 0.283 to 1.209 and were lower than those in *L.* × *formolongi*, which exhibited the lowest value of 1.368 for PC-3p-3100_2399. In addition, PC-3p-67_108977 was the most stable gene in *L.* × *formolongi* under different biotic and abiotic stresses. miR399a and miR5083 were identified as the most stable genes under heat stress and *B. elliptica* treatment in *L. regale*. Furthermore, under *B. elliptica* treatment, 5.8S was the most unstable gene among the candidates in both lilies. Hence, PC-3p-67_108977 and miR5083 were identified as the best genes for normalization in two kinds of lilies under four sets of experimental conditions. Overall, PC-3p-67_108977 and 5S were the most stable reference genes for all samples (Table 6).

Table 2
Expression stability ranking of candidate reference genes under different experiment conditions in *L.* × *formolongi* and *L. regale* calculated using NormFinder

	Rank	Developmental stages		Tissues		Heat stress		*B. elliptica* treatment		Total	
		Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
L. × formolongi	1	miR399a	0.920	PC-3p-3100_2399	1.368	PC-3p-67_108977	0.491	PC-3p-67_108977	0.732	PC-3p-67_108977	0.488
	2	PC-3p-3100_2399	1.058	PC-3p-67_108977	1.760	PC-3p-3100_2399	0.549	miR399a	0.737	5S	0.532
	3	miR2916	1.251	U6	1.805	miR2916	0.591	PC-3p-3100_2399	0.833	miR2916	0.543
	4	PC-3p-67_108977	1.286	miR399a	1.949	miR399a	0.675	miR5083	1.203	PC-3p-3100_2399	0.670
	5	5S	1.503	5S	2.123	5.8S	0.710	U6	1.558	U6	0.755
	6	miR5083	1.506	miR2916	2.519	U6	0.849	5S	2.448	miR399a	0.812
	7	5.8S	1.684	miR5083	2.926	5S	0.893	miR2916	2.699	miR5083	1.256
	8	U6	2.384	5.8S	3.327	miR5083	1.185	5.8S	3.054	5.8S	1.543
L. regale	1	U6	0.311	5.8S	0.283	miR399a	1.138	miR5083	0.081	miR5083	0.514
	2	miR2916	0.370	PC-3p-67_108977	0.723	miR2916	1.205	miR2916	0.100	PC-3p-3100_2399	0.654
	3	miR399a	0.435	5S	0.770	U6	1.309	PC-3p-67_108977	0.234	miR2916	0.724
	4	5S	0.851	miR399a	0.792	5.8S	1.320	miR399a	0.268	5S	0.946
	5	5.8S	0.873	U6	0.817	PC-3p-67_108977	1.711	PC-3p-3100_2399	0.335	U6	1.017
	6	PC-3p-3100_2399	1.055	PC-3p-3100_2399	0.935	PC-3p-3100_2399	1.766	5S	0.418	5.8S	1.058
	7	miR5083	1.092	miR5083	0.937	5 s	1.894	U6	0.529	miR399a	1.083
	8	PC-3p-67_108977	1.206	miR2916	1.209	miR5083	1.932	5.8S	1.602	PC-3p-67_108977	1.143

BestKeeper analysis

Based on the standard deviation (SD) and coefficient of variation (CV) calculated using BestKeeper, the stability rankings of the candidates in various samples were obtained and are presented in Table 3. According to the SD values, the most reliable gene was miR399a, which presented the lowest value not only during different developmental stages in *L.* × *formolongi* but also in different tissues of both lilies. PC-3p-67_108977 showed the highest stability under heat stress in *L.* × *formolongi* and *L. regale*. Additionally, under *B. elliptica* treatment, miR5083 and miR2916 were ranked as the top genes in *L.* × *formolongi* and *L. regale*, respectively, followed by miR399a and PC-3p-67_108977 in *L.* × *formolongi* and miR5083 and 5S in *L. regale*. Among all experiments, the highest SD value was observed for miR5083 under heat stress in *L.* × *formolongi* and *L. regale* and in different tissues of *L.* × *formolongi*. Considering all conditions tested in

this study together, PC-3p-67_108977 and miR399a were identified as the best reference genes in *L.* × *formolongi* and *L. regale*, respectively. A comprehensive analysis of all samples indicated that U6 and PC-3p-67_108977 were the best reference genes for lily (Table 6).

Table 3
Expression stability ranking of candidate reference genes under different experimental conditions in *L.* × *formolongi* and *L. regale* calculated using BestKeepe

	Rank	Developmental stages		Tissues		Heat stress		*B. elliptica* treatment		Total	
		Gene	Stability (CV ±SD)	Gene	Stability (CV ±SD)	Gene	Stability (CV ±SD)	Gene	Stability (CV ±SD)	Gene	Stability (CV ±SD
L. ×formolongi	1	miR399a	2.25 ± 0.69	miR399a	4.47 ± 1.29	PC-3p-67_108977	2.04 ± 0.53	miR5083	3.81 ± 1.09	PC-3p-67_108977	4.02 ± 1.05
	2	PC-3p-3100_2399	3.71 ± 1.09	PC-3p-3100_2399	4.98 ± 1.42	PC-3p-3100_2399	1.97 ± 0.60	miR399a	4.28 ± 1.21	5S	7.33 ± 1.82
	3	PC-3p-67_108977	4.28 ± 1.12	PC-3p-67_108977	5.47 ± 1.46	miR399a	2.21 ± 0.67	PC-3p-67_108977	4.82 ± 1.27	miR399a	7.66 ± 2.20
	4	5S	5.48 ± 1.34	5S	7.5 ± 1.93	miR2916	3.90 ± 1.01	PC-3p-3100_2399	5.15 ± 1.41	miR2916	9.20 ± 2.57
	5	miR2916	6.59 ± 1.66	U6	7.64 ± 2.27	5.8S	3.80 ± 1.12	miR2916	6.70 ± 1.51	U6	8.44 ± 2.39
	6	miR5083	9.37 ± 2.17	5.8S	9.70 ± 2.97	U6	4.11 ± 1.20	U6	5.67 ± 1.57	PC-3p-3100_2399	9.21 ± 2.57
	7	5.8S	8.48 ± 2.70	miR2916	13.58 ± 3.46	5S	5.02 ± 1.27	5.8S	8.12 ± 2.42	5.8S	11.74 ± 3.33
	8	U6	13.60 ± 3.57	miR5083	11.90 ± 3.70	miR5083	6.37 ± 1.48	5S	10.11 ± 2.43	miR5083	16.69 ± 4.55
L. regale	1	PC-3p-3100_2399	1.71 ± 0.42	miR399a	1.31 ± 0.29	PC-3p-67_108977	9.92 ± 2.17	miR2916	1.66 ± 0.49	miR399a	9.92 ± 2.22
	2	5.8S	2.56 ± 0.54	PC-3p-67_108977	1.43 ± 0.29	miR2916	10.67 ± 2.59	miR5083	2.12 ± 0.58	U6	8.74 ± 2.38
	3	5S	8.01 ± 1.67	PC-3p-3100_2399	2.21 ± 0.52	PC-3p-3100_2399	16.00 ± 4.19	5S	5.20 ± 1.10	miR5083	11.3 ± 2.98
	4	miR399a	7.29 ± 1.74	miR5083	3.04 ± 0.71	miR399a	17.59 ± 4.40	PC-3p-3100_2399	4.10 ± 1.28	miR2916	12.51 ± 3.24
	5	miR2916	7.01 ± 1.78	5.8S	5.03 ± 1.04	5S	27.12 ± 4.56	PC-3p-67_108977	4.46 ± 1.40	5S	20.09 ± 3.74
	6	U6	6.69 ± 1.88	U6	6.57 ± 1.77	5.8S	19.82 ± 4.67	U6	6.44 ± 1.66	PC-3p-3100_2399	14.01 ± 3.82
	7	miR5083	10.97 ± 3.09	5S	13.15 ± 1.82	U6	16.80 ± 4.97	miR399a	8.82 ± 1.77	5.8S	18.77 ± 4.62
	8	PC-3p-67_108977	12.84 ± 3.19	miR2916	11.33 ± 2.47	miR5083	21.97 ± 5.84	5.8S	9.97 ± 2.91	PC-3p-67_108977	18.49 ± 4.75

Delta CT method and RankAggreg analysis

The simple ΔCt approach was also used to compare the relative expression levels (Cq values) of 'pairs of genes' within every individual sample. These candidates were ranked according to the deviation in ΔCt in the two kinds of lilies (Additional file 3: Fig. S3). There were differences in the stability rankings under varied circumstances. In *L.* × *formolongi*, 5.8S was suggested to be the most stable gene, and U6 was the least reliable gene during development (Table 4). By contrast, U6 exhibited the most uniform expression level under biotic stress. PC-3p-3100_2399 and 5S were found to be reliable genes in different tissues and under abiotic stress, respectively. U6 also performed well under both biotic and abiotic stress conditions in *L. regale*. miR5083 and 5.8S were identified as the most suitable genes during development and in different tissues of *L. regale*. miR399a was identified as stably expressed in both *L.* × *formolongi* and *L. regale* under all tested conditions. For all samples, miR5083 was indicated to be the best reference gene.

Table 4
Expression stability ranking of the 8 candidate reference genes under different conditions in *L.* × *formolongi*

Method	1	2	3	4	5	6	7	8
(A) Developmental stages								
geNorm	PC-3p-3100_2399	miR399a	PC-3p-67_108977	5S	miR2916	miR5083	5.8S	U6
Normfinder	miR399a	PC-3p-3100_2399	miR2916	PC-3p-67_108977	5S	miR5083	5.8S	U6
BestKeeper	miR399a	PC-3p-3100_2399	PC-3p-67_108977	5S	miR2916	miR5083	5.8S	U6
Delta CT	5.8S	PC-3p-67_108977	5S	miR399a	PC-3p-3100_2399	miR2916	miR5083	U6
RankAggreg	miR399a	PC-3p-3100_2399	PC-3p-67_108977	5S	miR2916	miR5083	5.8S	U6
(B) Tissues								
geNorm	PC-3p-3100_2399	PC-3p-67_108977	U6	miR399a	5S	miR2916	miR5083	5.8S
Normfinder	PC-3p-3100_2399	PC-3p-67_108977	U6	miR399a	5S	miR2916	miR5083	5.8S
BestKeeper	miR399a	PC-3p-3100_2399	PC-3p-67_108977	5S	U6	5.8S	miR2916	miR5083
Delta CT	PC-3p-3100_2399	PC-3p-67_108977	miR399a	miR5083	U6	miR2916	5S	5.8S
RankAggreg	PC-3p-3100_2399	PC-3p-67_108977	miR399a	U6	5S	miR2916	miR5083	5.8S
(C) Heat Stress								
geNorm	PC-3p-67_108977	PC-3p-3100_2399	miR399a	miR2916	5.8S	U6	5S	miR5083
Normfinder	PC-3p-67_108977	PC-3p-3100_2399	miR2916	miR399a	5.8S	U6	5S	miR5083
BestKeeper	PC-3p-67_108977	PC-3p-3100_2399	miR399a	miR2916	5.8S	U6	5S	miR5083
Delta CT	5S	PC-3p-67_108977	miR399a	miR2916	5.8S	U6	PC-3p-3100_2399	miR5083
RankAggreg	PC-3p-67_108977	PC-3p-3100_2399	miR399a	miR2916	5.8S	U6	5S	miR5083
(D) B. elliptica treatment								
geNorm	PC-3p-67_108977	miR399a	PC-3p-3100_2399	miR5083	U6	5S	miR2916	5.8S
Normfinder	PC-3p-67_108977	miR399a	PC-3p-3100_2399	miR5083	U6	5S	miR2916	5.8S
BestKeeper	miR5083	miR399a	PC-3p-67_108977	PC-3p-3100_2399	miR2916	U6	5.8S	5S
Delta CT	U6	miR2916	miR399a	PC-3p-3100_2399	5S	5.8S	PC-3p-67_108977	miR5083
RankAggreg	PC-3p-67_108977	PC-3p-3100_2399	miR2916	miR399a	5.8S	U6	5S	miR5083
(E) Total								
geNorm	U6	miR399a	PC-3p-3100_2399	5S	miR2916	PC-3p-67_108977	5.8S	miR5083
Normfinder	PC-3p-67_108977	5S	miR2916	PC-3p-3100_2399	U6	miR399a	miR5083	5.8S
BestKeeper	PC-3p-67_108977	5S	miR399a	miR2916	U6	PC-3p-3100_2399	5.8S	miR5083
Delta CT	miR399a	PC-3p-3100_2399	PC-3p-67_108977	miR2916	5S	U6	5.8S	miR5083

Method	1	2	3	4	5	6	7	8
RankAggreg	PC-3p-67_108977	miR399a	5S	miR2916	PC-3p-3100_2399	U6	5.8S	miR5083

Ultimately, the results generated by the above methods were further analysed using the R package RankAggreg to determine the comprehensive rankings of the candidates (Additional file 4: Fig. S4). In *L.* × *formolongi*, the candidate reference miRNAs were more stable than traditional housekeeping genes according to our systematic analyses (Table 4). During development, miR399 and PC-3p-3100_2399 were the two most stable genes among the tested genes. PC-3p-3100_2399 was also suitable for use in different tissues. PC-3p-67_108977 exhibited the best stability under the stress treatments and was identified as an appropriate gene under all of the tested experimental conditions. However, PC-3p-67_108977 was the least stable gene across all of the tested conditions except in different tissues and under heat stress conditions in *L. regale* (Table 5). According to the RankAggreg package, U6 was the most stable gene during development and under biotic stress treatment. Moreover, 5.8S and miR399a were the most stable genes in different tissues and under heat stress, respectively. miR399a was the top-ranked gene according to RankAggreg analysis among all tested samples of *L. regale*. Overall, PC-3p-67_108977 and U6 were ranked as the best reference genes for the samples across all experimental conditions tested in the two lilies (Table 6).

Table 5
Expression stability ranking of the 8 candidate reference genes under different conditions in *L. regale*

Method	1	2	3	4	5	6	7	8
(A) Developmental stages								
geNorm	miR2916	U6	miR399a	5.8S	PC-3p-3100_2399	PC-3p-67_108977	5S	miR5083
Normfinder	U6	miR2916	miR399a	5S	5.8S	PC-3p-3100_2399	miR5083	PC-3p-67_108977
BestKeeper	PC-3p-3100_2399	5.8S	5S	miR399a	miR2916	U6	miR5083	PC-3p-67_108977
Delta CT	miR5083	miR2916	miR399a	5.8S	U6	PC-3p-3100_2399	PC-3p-67_108977	5S
RankAggreg	U6	miR2916	miR399a	5.8S	PC-3p-3100_2399	5S	miR5083	PC-3p-67_108977
(B) Tissues								
geNorm	PC-3p-67_108977	miR399a	PC-3p-3100_2399	miR5083	5.8S	U6	5S	miR2916
Normfinder	5.8S	PC-3p-67_108977	5S	miR399a	U6	PC-3p-3100_2399	miR5083	miR2916
BestKeeper	miR399a	PC-3p-67_108977	PC-3p-3100_2399	miR5083	5.8S	U6	5S	miR2916
Delta CT	5.8S	5S	miR2916	U6	miR399a	PC-3p-67_108977	PC-3p-3100_2399	miR5083
RankAggreg	5.8S	PC-3p-67_108977	miR399a	PC-3p-3100_2399	U6	miR5083	5S	miR2916
(C) Heat Stress								
geNorm	miR399a	5.8S	U6	miR5083	miR2916	PC-3p-67_108977	PC-3p-3100_2399	5S
Normfinder	miR399a	miR2916	U6	5.8S	PC-3p-67_108977	PC-3p-3100_2399	5S	miR5083
BestKeeper	PC-3p-67_108977	miR2916	PC-3p-3100_2399	miR399a	5S	5.8S	U6	miR5083
Delta CT	U6	miR399a	5.8S	miR5083	miR2916	5S	PC-3p-3100_2399	PC-3p-67_108977
RankAggreg	miR399a	PC-3p-3100_2399	PC-3p-67_108977	5S	miR2916	miR5083	5.8S	U6
(D) B. elliptica treatment								
geNorm	miR5083	miR2916	5S	PC-3p-3100_2399	PC-3p-67_108977	U6	miR399a	5.8S
Normfinder	miR5083	miR2916	PC-3p-67_108977	miR399a	PC-3p-3100_2399	5S	U6	5.8S
BestKeeper	miR2916	miR5083	5S	PC-3p-3100_2399	PC-3p-67_108977	U6	miR399a	5.8S
Delta CT	U6	PC-3p-67_108977	miR2916	miR399a	miR5083	5S	PC-3p-3100_2399	5.8S
RankAggreg	U6	miR399a	miR2916	miR5083	5S	PC-3p-3100_2399	5.8S	PC-3p-67_108977
Total								
geNorm	miR5083	miR399a	U6	5S	miR2916	PC-3p-3100_2399	PC-3p-67_108977	5.8S
Normfinder	miR5083	PC-3p-3100_2399	miR2916	5S	U6	5.8S	miR399a	PC-3p-67_108977
BestKeeper	miR399a	U6	miR5083	miR2916	5S	PC-3p-3100_2399	5.8S	PC-3p-67_108977
Delta CT	miR399a	U6	5.8S	miR5083	PC-3p-3100_2399	5S	miR2916	PC-3p-67_108977

Method	1	2	3	4	5	6	7	8
RankAggreg	miR399a	U6	miR5083	5S	miR2916	PC-3p-3100_2399	5.8S	PC-3p-67_108977

Table 6
Expression stability ranking of the 8 candidate reference genes in all samples from two lilies

Methods	1	2	3	4	5	6	7	8
geNorm	PC-3p-67_108977	U6	5S	miR2916	PC-3p-3100_2399	miR399a	miR5083	5.8S
Normfinder	PC-3p-67_108977	5S	miR2916	PC-3p-3100_2399	U6	miR399a	miR5083	5.8S
BestKeeper	U6	PC-3p-67_108977	miR2916	PC-3p-3100_2399	5 s	miR5083	399a	5.8S
Delta CT	miR5083	miR2916	U6	PC-3p-67_108977	399a	5 s	PC-3p-3100_2399	5.8S
RankAggreg	PC-3p-67_108977	U6	miR2916	PC-3p-3100_2399	5S	miR399a	miR5083	5.8S

Discussion

The analysis of miRNA expression is important for investigating miRNA function in biological research. The reference genes employed for such analyses are required to exhibit relatively stable expression profiles across various biological processes [42]. Compared with similar studies related to mRNA qRT-PCR, there have been only a few investigations of the stability of multiple reference genes for miRNA qRT-PCR in plant species such as soybean [19], castor bean [23], citrus [43] and peach [44]. In lily, the reliability of candidate genes has been validated only during somatic embryogenesis [8]. However, the stability of miRNAs under different conditions, particularly during the flowering induction process, which involves multiple flowering pathways integrated by various miRNAs, has not been confirmed in different lilies.

In reference gene evaluation studies, researchers choose different genes as candidate reference genes to expand the selection range to obtain the most optimal genes. A majority of prior studies have shown that potential miRNAs exhibit more consistent expression levels than classic reference genes under certain conditions in multiple plants, such as soybean [19], longan [24] and wheat [45]. In this study, similar results were obtained mainly in *L.* × *formolongi*. PC-3p-67_108977 and miR399a were recommended as the best reference genes for all samples of *L.* × *formolongi*. In *L. regale*, miR399a and U6 were identified as the most suitable genes. PC-3p-67_108977 exhibited unstable expression in some conditions, such as different developmental stages and *B. elliptica* treatment experiments. Therefore, we concluded that the genotype, the experimental conditions, and even the source of the candidate miRNA sequence could influence the expression stability of these candidates. Moreover, two novel miRNAs were more stable than most conserved miRNAs across various conditions in *L.* × *formolongi* but displayed less stable expression in *L. regale*. We suggest that the differences in performance may be due to the low evolutionary conservation and species specificity of these genes.

The traditional housekeeping genes U6, 5S and 5.8S showed differences in expression stability in the two lilies. U6, a common reference gene that is widely used for mRNA and miRNA normalization in qRT-PCR, shows stable expression during plant regeneration in poplar and under salinity and cold stress in grapevine [46, 47]. In the present study, this gene exhibited the most stable expression during *L. regale* development and presented less stable expression under the other treatments applied in this study. The finding that 5S was relatively unstable under different treatments was consistent with previous results obtained for this gene during citrus somatic embryogenesis [43] and peach [44]. 5.8S has been selected as a reference gene for normalizing target gene expression [48, 49], but its stability has rarely been evaluated under different conditions. In this study, 5.8S was one of the most unstable genes under most of the tested conditions, whereas 5.8S ranked first in various tissues of *L. regale*, indicating its variable expression under most conditions.

There was variation between the stability rankings generated by different algorithms, and discrepancies have also been observed in other plants, as found for miRNA in common wheat [50] and mRNA in peanut [51] and papaya [52]. In this study, the geNorm and NormFinder results showed a striking similarity, but they were slightly different from the ranking generated by BestKeeper. The results differed from those obtained during *Lilium* somatic embryogenesis, for which the results of BestKeeper were similar to those of geNorm [8], but they were in agreement with the results obtained in potato under abiotic stress [20]. In fact, it is common to find differences among the rankings generated by the algorithms due to the different calculation approaches applied and their sensitivity to co-regulated candidates. Hence, given the advantages and characteristics of each approach, it is necessary to adopt multiple methods to investigate the reliability of reference genes for gene expression studies.

The optimal number of reference genes according to the geNorm algorithm is determined through pairwise variation (V). In our study, the parameter Vn/ Vn + 1 scores were higher than the recommended cut-off value (0.15). This result is rare but was also observed in *Cephalotaxus hainanensis* under various stimuli [53]. Thus, we did not consider the recommended V score to be suitable for our experimental system, and we found that one reference gene was ideal for normalizing the samples in this study.

Conclusion

In conclusion, the stability of 8 candidate reference genes, including 3 classic housekeeping genes and 5 miRNAs selected from small RNA libraries with stable high expression levels during *L.* × *formolongi* development, was evaluated under different circumstances, including different developmental stages, tissues and stresses, in *L.* × *formolongi* and *L. regale* using multiple algorithms. The analyses showed that the novel miRNAs PC-3p-67_108977 and miR399a

or miR399a and U6 were the most stable genes for *L.* × *formolongi* or *L. regale*, respectively, under all tested experimental conditions. PC-3p-67_108977 and U6 were identified as appropriate reference genes for miRNA qRT-PCR studies in lily. The results of this study will facilitate accurate miRNA expression normalization in studies of miRNA function in lily.

Abbreviations

miRNAs: MicroRNAs; qRT-PCR: quantitative real time polymerase chain reaction; SnRNA: small nuclear; RNA; rRNA: Ribosomal RNA; 18S: 18S Ribosomal RNA; 5S: 5S Ribosomal RNA; 5.8S: 5.8 Ribosomal RNA; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; hpi: postinoculation; Cq: RT-qPCR-derived quantification; MFEIs: minimum folding free energy indexes

Declarations

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Not applicable.

Authors' contributions

QZ and GJ designed the study. QZ, XG, LW and YZ conducted the experiments. QZ performed the data analysis and wrote the pater. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets supporting the conclusions and description of a complete protocol are included within the article

Ethics approval and consent to participate

Not applicable.

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Consent for publication

All authors have consented to this publication.

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Figures

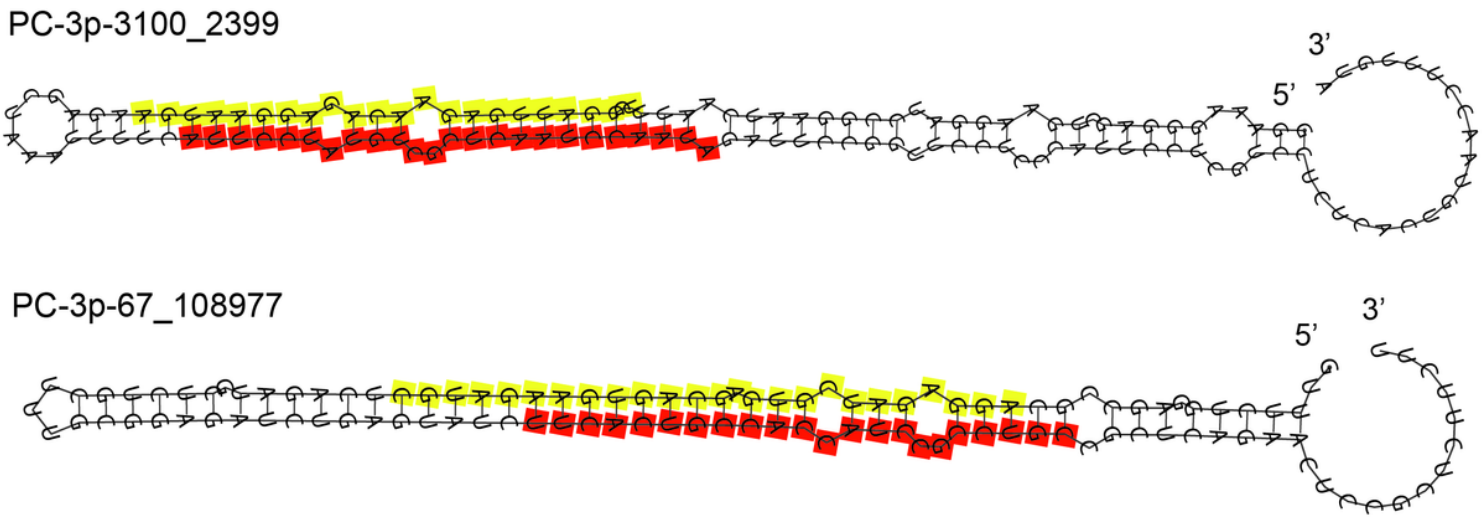

Figure 1

Secondary structure predictions for precursors of novel miRNAs selected as reference genes in lily. The red and yellow colored sequences represent mature miRNAs and miRNA* respectively

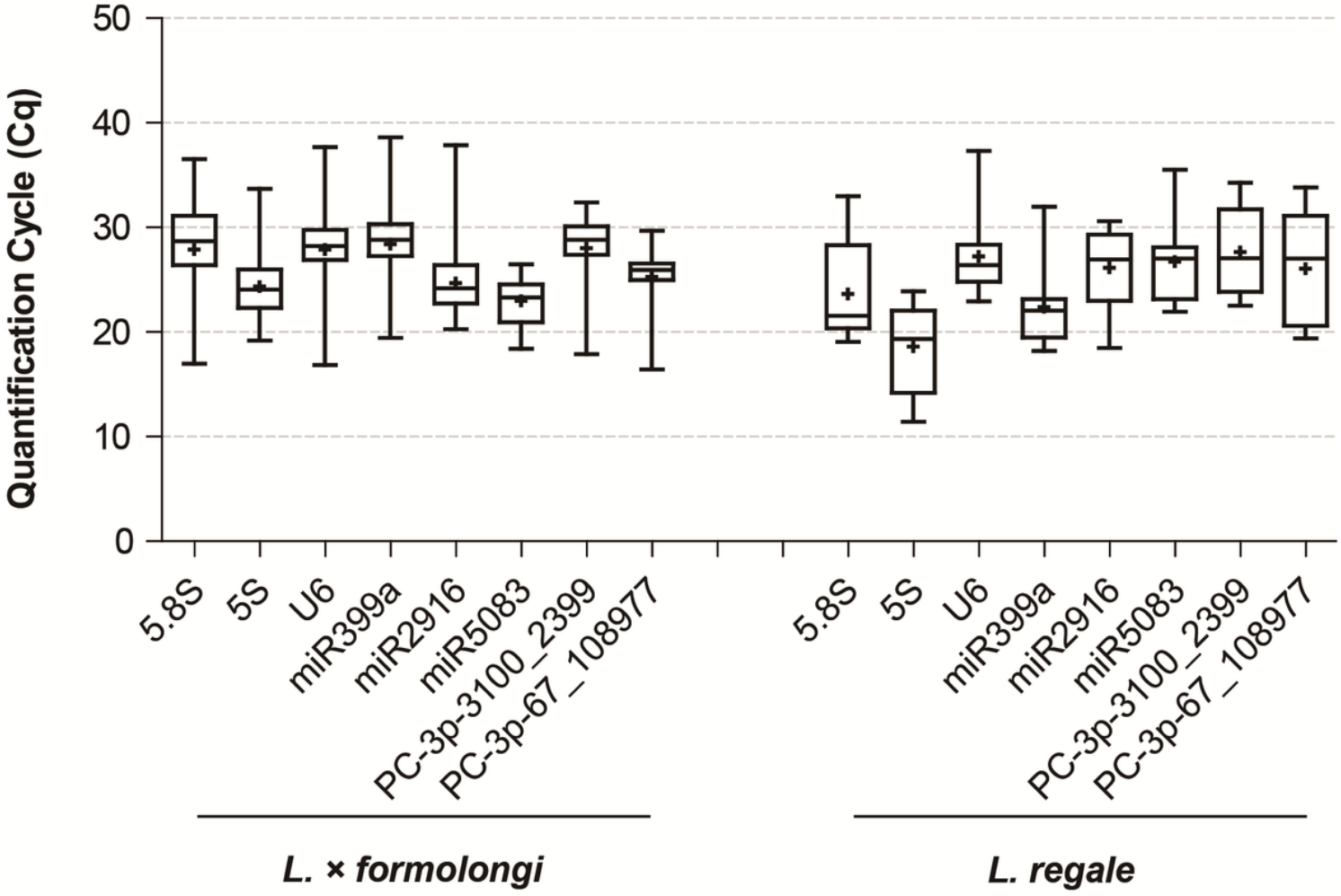

Figure 2

Box plots of the Cq values of eight candidate reference genes under all conditions in L. × formolongi and L. regale. A line across the box denotes the median, and the box denotes the first and third quartile. The whisker caps mean the minimum and maximum values

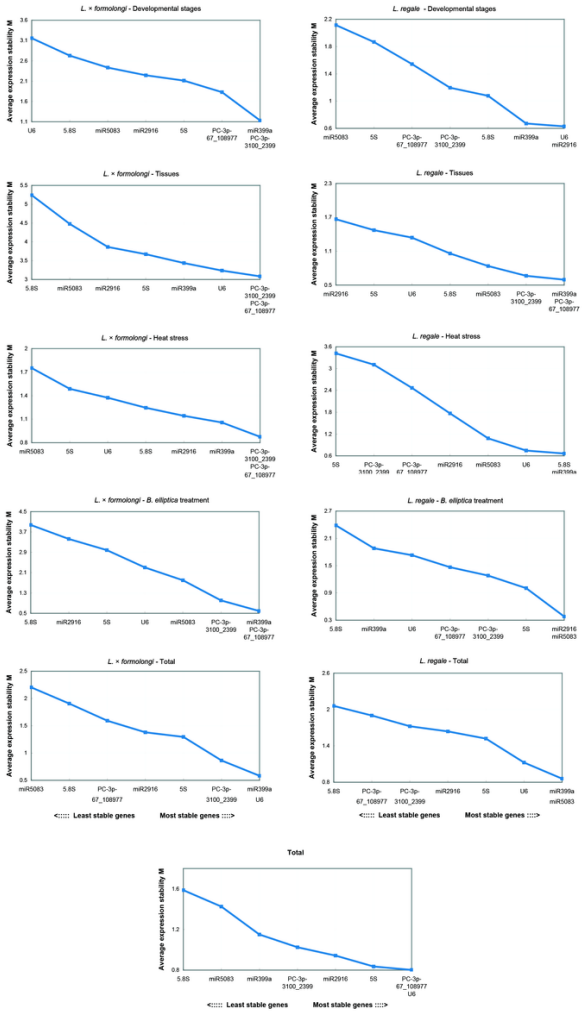

Figure 3

Average expression stability (M) value and ranking of eight candidate reference genes in two lilies obtained using geNorm

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