



## Validation of Housekeeping Genes as Internal Control for Studying Gene Expression in *Rhododendron Molle* G. Don Located on Dabie Mountains

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**Abstract** *Rhododendron molle* G. Don, a member of the family of Rhododendron, possesses high ornamental and medicinal value. As a species specific to Dabie Mountains, its distribution was scattered. The estimation of expression stability of internal reference genes was vital for molecular biological and genetic research. In this study, expression pattern of candidate genes *RmRPL3-1*, *RmNAC-1*, *RmCOX1-4*, *RmRmPDS-1*, *RmUBQ-6*, and *RmL10-4* were analyzed in different tissues of *R. molle* by real-time quantitative RT-PCR, including root, stem, leaf, and flower. According to GeNorm, NormFinder, and Best-keepers softwares, the most stable internal reference in different tissues of *R. molle* was the combination of *RmCOX1-4* and *RmUBQ-6*. The most stable internal reference genes selected in this study will provide a basis for the following gene expression study in *R. molle*.

**Keywords** *Rhododendron molle* G. Don; Reference genes; qRT-PCR; Expression pattern

*Rhododendron molle* G. Don, also known as goat blossom and lamb staggering, belongs to the genus of *Rhododendron* (Ericaceae), which is mainly distributed along the Yangtze River (Zhou et al., 2017). *R. molle*, having large crown and funnel-shaped flower, is a specific ornamental species unique to China. Moreover, *R. molle* also has extremely high medicinal value in treating rheumatoid arthritis, injury, enhancing muscles, activating collaterals. Due to strong insecticidal efficacy, the flowers, leaves, and branches have been traditionally used in forestry protection (Klocke et al., 1991; Chen and Hu, 1999). Recently, more research were focused on rapid propagation in vitro, clarification of insecticidal mechanism, and genetic diversity of the green pesticide *R. molle*, but less on analysis of gene expression profiling (Li et al., 2018; Xiao et al., 2018).

As a common technique in molecular biology, quantitative real time-fluorescence polymerase chain reaction (qRT-PCR) was widely used in the quantitative analysis of target genes due to the characteristics of high sensitivity, good accuracy, strong specificity, simple operation, rapid, and efficient (Xiao et al., 2016). However, stable internal reference genes were critical for the qRT-PCR amplification, which could eliminate errors generated during qRT-PCR process (Fan et al., 2018). The best reference genes are housekeeping genes, whose expression level was never affected by tissue types, developmental stages, and external factors (Bustin and Nolan, 2004; Han et al., 2012). However, expression patterns of genes are diverse, as functions of genes are different in different tissues, at different developmental stages, and even under various environments (Tashiro et al., 2016; Song et al., 2017). Therefore, before studying the dynamic expression pattern of target genes, it is necessary to evaluate the stability of candidate reference genes according to specific experimental conditions and plant development stage, select the reference genes with the most stable expression level, so as to maximize the accuracy of quantitative gene research. In this study, six candidate internal reference genes were validated in different tissue of *R. molle* (root, stem, leaf, and flower), a species specific to Dabie Mountains. The most stable internal reference in different tissues were screened out according to GeNorm, NormFinder, and Best-keeper softwares, which will provide a basis for the following gene expression study in *R. molle*.

## 1 Results and Analysis

### 1.1 Quality detection of total RNA in root, stem, leaf, and flower of *R. molle*

Total RNA was extracted from root, stem, leaf, and flower of *R. molle*, and was analyzed by agarose gelelectrophoresis. The bands of 28SrRNA and 18SrRNA were very intact, and the brightness of 28SrRNA was twice of that of 18SrRNA without obvious RNA degradation or genomic DNA contamination. The OD260/OD280 ratios of four tissue RNA samples were all within the range of 1.8~2.0 by ultraviolet visible spectrophotometer, indicating that the purity of total RNA was high enough for subsequent qRT-PCR amplification (Figure 1).

### 1.2 Evaluation of candidate reference gene

The size of amplification products of six candidate reference genes ranged from 200 bp to 230 bp, and annealing temperature for these specific primer pairs was within the range of 52°C~60°C. DNA pool was mixed with equal portions of DNA extracted from root, stem, leaf, and flower. Then, PCR amplification was performed with the DNA pool to validate the specificity of primer pairs. Results showed that length of the amplified products was consistent with the expectation, and all were single bands without non-specific amplification. The expression level of these six candidate genes was stable in root, stem, leaf and flower tissue. The melting curve showed the specific expression of six candidate genes, and specific bands were also detected in agarose gel electrophoresis. The linear correlation coefficients  $R^2$  calculated according to the standard curve were all greater than 0.99, and the amplification efficiency ranged from 95.0% to 105.0%. These primer pairs met the requirements of qRT-PCR, which could be used for stability evaluation of reference genes (Table 1).

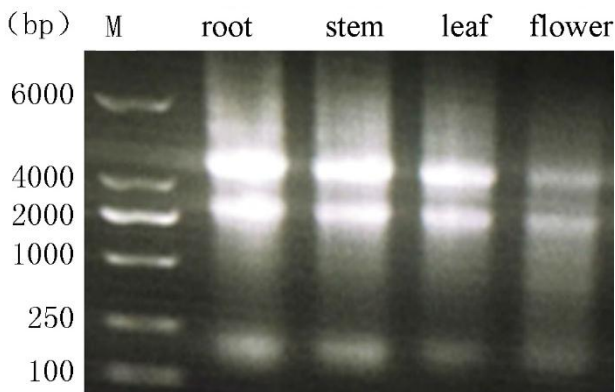


Figure 1 RNA extracted from four different tissues of *R. molle*

Table 1 Primer information of six reference genes

Gene name	Forward/Reverse primer sequence (5'-3')	Annealing temperature (°C)	Product length (bp)	Amplification efficiency (%)	Correlation coefficient ( $R^2$ )
<i>RmCOX1</i>	F:GGTCCGTTCTAGTGACAGCA R:GATACCGGATCCAGGCAGAA	60	203	103.05	0.994
<i>RmNAC-1</i>	F:TGGAACTCGGAAGCGTAGAA R:TTCTCCAGTGCCCAAGTGAT	58	205	99.76	0.992
<i>RmPDS-1</i>	F:ACGAATTGCTTGCTTCCCG R:GCACTCTTAGGGATTGCTGTC	57	216	101.12	0.995
<i>RmUBQ-6</i>	F:TGTGAAGACTCTTACTGGGAAG R:CTCCCATCTCCAAGTCTT	60	229	98.69	0.997
<i>RmL10-4</i>	F:AGCAGGGAAGACGAAGATCA R:TAGCCAAGCGTTCATACCCT	52	220	99.24	0.994
<i>RmRPL3</i>	F:CATCTGAGCGAGGAGGTGAA R:GGTGTGCCTTCTTCTGCTTC	58	217	99.73	0.996

### 1.3 Expression level of candidate reference genes

The *Ct* values of *RmCOX1* ranged between 21.15 and 26.41 in different tissues, with the mean value of 22.97. In particular, the maximum *Ct* value of *RmCOX1* was observed in stem, while the minimum value was in leaf. *Ct* values of *RmNAC-1* ranged from 21.80 (stem) to 28.95 (flower) with the mean *Ct* value of 24.05. Moreover, *Ct* values of *RmL10* ranged from 31.21 (root) to 32.87 (stem) with the mean value of 32.04. The *Ct* values of *RmPDS-1* and *RmUBQ-6* were within ranges of 20.73~27.46 and 30.42~35.11, with the average value of 23.36 and 31.76, respectively. *Ct* values of *RmRPL3* varied from 20.57 to 27.11, with the mean value of 22.68. In related to candidate reference gene *RmPDS-1*, *RmUBQ-6*, and *RmRPL3*, the maximum *Ct* value was observed in the stem and the minimum *Ct* value existed in flower.

### 1.4 Expression stability of the candidate reference genes

Analyzing the stability of six candidate reference genes with geNorm, the *Ct* value was converted into relative expression quantity *Q*, which was used to calculate the expression stability *M*. The smaller the *M* value, the higher the stability of the internal reference gene (Stamova et al., 2009). The expression stability (from high to low) of the six candidate genes was as follow: *RmRPL3-1*>*RmNAC-1*>*RmCOX1-4*>*RmPDS-1*>*RmUBQ-6*>*RmL10-4*. The optimal genes screened were the combination of *RmRPL3-1* and *RmNAC-1*, while the worst was *RmL10-4* (Figure 2; Table 2).

The geNorm software was also used to calculate the paired difference value *V*, which could determine the optimal number of internal reference genes. Results showed that the threshold value of the paired difference value was 0.15. If  $V_{n/n+1}$  was less than 0.15, *n*+1 internal reference gene were needed to correct the expression of the target gene (Jiang et al., 2017). In this study, the  $V_{2/3}$ ,  $V_{3/4}$ ,  $V_{4/5}$  and  $V_{5/6}$  were all greater than 0.15, as they were 0.315, 0.214, 0.207 and 0.543, respectively. Therefore, the combination number of suitable reference genes should be determined by classification.

Normfinder software was used to calculate the stable expression value (*S*) of candidate reference genes. Lower *S* values means higher stability (Michael et al., 2004). The *S* values of the six candidate reference genes ranged from 0.22 to 2.25. *RmUBQ-6* gene had the lowest *S* value but was the most stable reference gene. However, the highest *S* value and the least stable gene was *RmL10-4*. According to the *S* value, stability of the six candidate internal reference genes was as follow: *RmUBQ-6*>*RmCOX-1*>*RmRPL3-1*>*RmPDS-1*>*RmNAC-1*>*RmL10-4* (Table 2). In particular, the stability of *RmL10-4* was the worst expression in different tissues both in geNorm and Normfinder analysis, which was not suitable for study on gene expression.

By the Bestkeeper software, the stability of reference genes were selected according to the SD and CV of *Ct* values, as well as coefficient of correlation (*r*) and its index. The most stably expressed reference gene had the *Ct* value with lowest SD and CV values. Genes with SD value less than 1 are suitable for internal reference genes (Yan et al., 2015). The candidate internal reference gene with high expression stability was *RmL10-4* (SD=0.61), while stability of *RmNAC-1* (SD=2.451) was the lowest (Table 2).

According to GeNorm, NormFinder, and Best-keeper softwares, the most stable internal reference in different tissues of *R. molle* was the combination of *RmCOX1-4* and *RmUBQ-6*, followed by *RmRPL3-1*. The worst reference gene were *RmL10-4* and *RmNAC-1*.

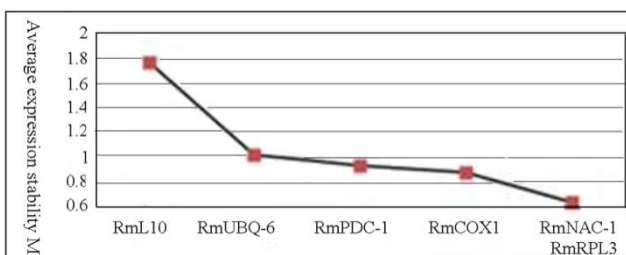


Figure 2 Expression stability of candidate reference genes in different tissues based on Genorm software

Table 2 Stability ranking of six candidate reference genes in different tissues

No.	GeNorm ranking	NormFinder ranking	BestKeeper ranking
1	<i>RmRPL3-1</i>	<i>RmUBQ-6</i> (S=0.224)	<i>RmL10-4</i> (SD= 0.61; CV=1.91)
2	<i>RmNAC-1</i>	<i>RmCOX1-4</i> (S=0.304)	<i>RmUBQ-6</i> (SD=1.68 ; CV=5.29)
3	<i>RmCOX1-4</i>	<i>RmRPL3-1</i> (S=0.554)	<i>RmCOX1-4</i> (SD= 1.72; CV=7.51)
4	<i>RmPDS-1</i>	<i>RmPDS-1</i> (S=0.688)	<i>RmRPL3-1</i> (SD=2.22 ; CV=9.78)
5	<i>RmUBQ-6</i>	<i>RmNAC-1</i> (S=0.804)	<i>RmPDS-1</i> (SD=2.33 ; CV=9.98)
6	<i>RmL10-4</i>	<i>RmL10-4</i> (S=2.247)	<i>RmNAC-1</i> (SD=2.45 ; CV=10.20)

## 2 Discussion

In this study, expression pattern of candidate genes *RmRPL3-1*, *RmNAC-1*, *RmCOX1-4*, *RmRmPDS-1*, *RmUBQ-6*, and *RmL10-4* were analyzed in different tissues (root, stem, leaf, and flower) of *R. molle* by real-time quantitative RT-PCR. Lengths of actin and 18S rRNA genes achieved from transcriptome data were 325 bp and 289 bp, respectively. No clear target bands were obtained even more than ten times had been tried. Therefore, Actin and 18S rRNA genes were not considered in the validation of candidate reference genes. At present, geNorm, NormFinder and BestKeeper were the most effective analysis software to evaluate the stability of reference genes, though they used different statistical methods. Therefore, comprehensive analysis is needed to screen the most suitable internal reference genes (Hu et al., 2010; Ponton et al., 2011). According to these three software, stable internal reference genes have been validated from *Chrysanthemum lavandulifolium*, *Zanthoxylum bungeanum*, *Robinia pseudoacacia* L., *Hemarthria compressa*, and *Ginkgo biloba* (Fei et al., 2018; Lin et al., 2019; Su et al., 2019).

Species belonging to *Rhododendron* play important roles in landscaping. However, research on molecular biology and genetics were limited, especially the validation of candidate reference genes. In *Rhododendron delavayi* Franch., the expression stability of 11 candidate reference genes (*GAPDH*, *Tubulina-5*, *Ubiquitin*, *Actin*, *EF1a*, *UEP*, *UEC11*, *UEC2*, Tubulin beta, TATA-binding protein, *TIP41-LIKE FAMILYPROTEIN*) were validated in leaves at different developmental stages and under drought stress with qBASE and NormFinder software (Zhang, 2019).

In this study, six candidate internal reference genes of *R. mole* were validated in different tissues. With geNorm software, the most stable genes were *RmRPL3-1* and *RmNAC-1*, while the worst was *RmL10-4*. Meanwhile, expression stability of *RmUBQ-6* was most stable with Normfinder analysis, but ranked five by geNorm analysis. Stability of *RmL10-4* varied greatly with geNorm, Normfinder, and Bestkeeper. Bestkeeper software showed that the expression stability of *RmNAC-1* was low, which was consistent with the results of Normfinder software.

In other research, stability of candidate reference genes also differed with different software, which might be caused by the different counting principles and methods of each software. Therefore, comprehensive analysis with several softwares should be performed to screen the best reference genes for gene expression profile analysis. Frequently, method of weighted assignment are adopted (Wang et al., 2019). According to GeNorm, NormFinder, and Best-keeper softwares, the most stable internal reference in different tissues of *R. molle* was the combination of *RmCOX1-4* and *RmUBQ-6*, while the worst was *RmPDS-1*.

In the research on gene expression profile of peach trees, reference gene *UBQ10* was the best, while *RPL13* was the least (Tong et al., 2009). The best reference gene was *RmUBQ-6*, while the poorest one was *RmRPL3-1*, which was consistent with the research of Tong et al. (2009).

*RPL* was the most stable reference gene in *Lablab purpureus* (Linn.) Sweet under abiotic stress (Ragini et al., 2019), but was not the suitable reference gene in molecular biology study of different tissues of *R. molle*. Moreover, *COX* was the most stable internal reference gene of *Chlamydomonas reinhardtii* under different culture conditions (Zhang, 2013). Similarly, stable expression of *COX* was also observed in different tissues of

*R. molle*. Reference genes vary greatly in different varieties, different tissue parts, at different developmental stages, and under different treatment conditions. Therefore, it is necessary to validate the expression stability of internal reference genes before used in quantitative analysis of gene expression.

### 3 Materials and Methods

#### 3.1 Materials

A 10-year-old *R. molle* plant was selected, which is preserved in the Rhododendron germplasm resource garden of Huanggang Normal University. During full flowering stage, fresh roots, stems, leaves, and flowers were collected and conserved at -80°C for further use.

#### 3.2 Extraction of total RNA and first-strand cDNA synthesis

Total RNA was extracted from roots, stems, leaves and flowers of *R. molle* with Plant RNA kit (OMEGA), and the quality and concentration were determined by UV spectrophotometer and agarose gel electrophoresis. The qualified RNA samples were reverse transcribed into first-strand cDNA with Reverse Transcriptase M-MLA (Takara, Dalian) and stored at -20°C for further use.

#### 3.3 Amplification of candidate internal reference genes and screening of gene-specific primers

Based on *R. molle* petal transcriptome data (NCBI accession number: SRR5247114), six candidate house-keeping genes were selected, including *RmRPL3*, *RmNAC-1*, *RmCOX1*, *RmPDS-1*, *RmUBQ-6*, and *RmL10*. The online software Primer 3.0 was used to design primer pairs in conservative gene region according to primer design principle of qRT-PCR. The cDNA pool was prepared by mixing different cDNA with equal proportion. After serial 2-fold dilutions (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128), these cDNA templates were used to verify gene-specific primer pairs. PCR amplification products were separated by 2% agarose gel electrophoresis. Primer pairs with specific amplification and clear amplification products were screened out.

#### 3.4 Analysis of expression level of candidate internal reference genes

The 10-fold diluted cDNA samples of roots, stems, leaves and flowers of *R. molle* were used as templates for qRT-PCR amplification. Particularly, 10  $\mu$ L qRT-PCR reaction system was set: 5  $\mu$ L 2 $\times$ SYBR Premix Ex *Taq*<sup>TM</sup> fluorescent dye (Takara), 2  $\mu$ L of cDNA templates, 0.4  $\mu$ L (10  $\mu$ M) of each primer, and 0.2  $\mu$ L of ROX Reference Dye, and 2.4  $\mu$ L sterilized deionized water. The thermal conditions included initial denaturation at 95°C for 10 min, 40 cycles (95°C for 15sec, 60°C for 60 sec), and 1 cycle (95°C for 30 sec, 60°C for 15 sec). In addition, melting curve was programmed to test the expected amplification product at 60°C~95°C. All experiments were performed three times for eliminating handling error.

#### 3.5 Comprehensive evaluation of the stability of candidate reference genes

GeNorm, NormFinder, and Best-keeper softwares were used to evaluate the stability of six candidate reference genes. In particular, the *Ct* value was calculated directly by BestKeeper. However, the transformed value ( $Q = E^{-(Ct_{\text{sample}} - Ct_{\text{min}})}$ ) was adopted by GeNorm and NormFinder. Combing these calculating results, the suitable reference genes were screened.

#### Authors' contributions

Zhang Yujia and Wang Shuzhen were executors of experimental design and the experiment. Shu Du, Dong Xue, and Du Ruichen performed data analysis, and wrote the draft paper. Li Zhiliang participated in experimental design and analysis of the experimental results. Wang Shuzhen was in charge of the project, guided experimental design, performed data analysis, and corrected the paper. All authors read and approved the final manuscript.

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