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Identification and validation of reference genes for gene expression studies in postharvest rose flower (*Rosa hybrida*)

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ABSTRACT

Optimal reference genes are important for data normalization so that accurate and reliable gene expression measurements may be obtained in both semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR (qRT-PCR) methods. This study firstly evaluated potential reference genes in petals of rose flower (*Rosa hybrida*) under postharvest stress conditions and in various floral organs during flower opening and senescence, combining both RT-PCR and qRT-PCR analysis. The expression stabilities of gene members from three traditional housekeeping gene families – actin (*RhACT*), tubulin (*RhTUB*) and ubiquitin (*RhUBI*) – were assessed using two analysis software packages, geNorm and NormFinder. The results showed that, for cut rose flower, the optimal reference genes were *RhUBI1* for dehydration treatment and receptacles; *RhTUB2* for exogenous ethylene; *RhACT4* for gibberellic and abscisic acid treatments, wounding and stamens; *RhUBI6* for petals; *RhUBI2* for sepals; and *RhACT1* for gynoecia, respectively. Our results provide guidelines for reference gene(s) selection under different postharvest conditions and point the way towards more accurate and widespread use of qRT-PCR in rose flower.

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1. Introduction

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involved in signaling and metabolic pathways in organisms. This is achieved by different techniques; Northern blotting, RT-PCR and qRT-PCR have been used frequently. Among these, RT-PCR has been one of the most widely adopted procedures (Tan and Weis, 1992). The quantification of gene expression via qRT-PCR is the most commonly used technique to date, owing to its outstanding accuracy, broad dynamic range, and sensitivity (Artico et al., 2010; Bustin, 2002; Wong and Medrano, 2005).

Regardless of the technique used for gene expression analysis, data normalization is crucial to obtain accurate and reliable ifferent normalization strategies al., 2005); the use of endogenous, unregulated reference gene transcripts is the most common method (Vandesompele et al., 2009; VanGuilder et al., 2008). The ideal reference genes (previously known as "housekeeping genes") act as internal controls. Their expression profiles should be stable and hold relatively high expression levels in different tissues and cell types under experimental conditions (Nolan et al., 2006; Wan et al., 2010). Generally, there is no gene that meets all requirements for every experimental condition. Misrepresentation of target gene expression may result from the unstable expression levels of conventional reference genes, including glyceraldehyde-3-phosphate dehydrogenase, α-tubulin, actin and 18S ribosomal RNA (Brunner et al., 2004; Czechowski et al., 2005; Ferguson et al., 2010; Radonic et al., 2004). Therefore, a systematic validation of the expression stability of candidate reference genes should be conducted in preliminary experiments, assessing their usefulness for gene expression normalization (Gutierrez et al., 2008; Nolan et al., 2006; Wan et al., 2010).

In recent years, there have been a number of studies on the validation of reference genes in different plant species, e.g. *Arabidopsis* (Czechowski et al., 2005), rice (Jain et al., 2006), tomato (Exposito-Rodriguez et al., 2008), potato (Nicot et al., 2005) and *Petunia* (Mallona et al., 2010). Most of these studies used a list of

Abbreviations: RT-PCR, semi-quantitative reverse transcription polymerase chain reaction; qRT-PCR, quantitative real-time RT-PCR; RhACT, actin of *Rosa hybrid*; *RhTUA* and *RhTUB*, α- and β-tubulin of *Rosa hybrid*; RhUBI, ubiquitin of *Rosa hybrid*; GA, gibberellic acid; ABA, abscisic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 18S *rRNA*, 18S ribosomal RNA; EB, ethidium bromide; EST, expressed sequence tag; *EF1a*, elongation factor1-alpha; CYP, cyclophilin.

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reference genes from other plant species and tested them under their own experimental conditions. Then, software-based applications such as geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) or BestKeeper (Pfaffl et al., 2004) were used to identify the best reference gene statistically from a group of candidate genes in a defined set of biological samples. Fewer studies have examined the use combining both RT-PCR and qRT-PCR analysis.

Roses are the most economically important ornamental plant and belong to the top five ornamentals worldwide. Cut roses account for about 21% and 31% of all cut flowers traded in China and in European auctions, respectively (Heinrichs, 2008). However, cut roses for commercial production are usually harvested at an open-bud stage and are extremely susceptible to damage by abiotic or biotic stresses during postharvest handling, such as dehydration, ethylene or wounding. Such stresses result in abnormal rose flower opening, including flower wilts, bent neck, and tightened buds and flowers (Jin et al., 2006; Xue et al., 2008). This has led to investigations into the effect of different stresses on flower opening and senescence at a physiological and biochemical level in cut roses over several decades worldwide (Kumar et al., 2008). In the breeding, cultivation and postharvest handling of cut rose flowers, breeders and researchers need to pay more attention to the importance of tolerance or resistance to stress conditions. Some candidate genes for these characteristics were recently identified by genetic and molecular analyses (Biber et al., 2010; Ma et al., 2008; Xue et al., 2009).

Previously, *PP2A*, *SAND* and *UBC* have been recommended as suitable reference genes, which, in different combinations, may be used for normalization in expression analyses via qRT-PCR for different rose tissues and stress treatments (Klie and Debener, 2011). However, there was little focus on the expression variation among the family gene members. To date, there has been no well-defined and validated set of reference genes described for rose flower (*Rosa hybrida*) that show stable expression across a range of postharvest stress conditions. In this study, we focused on three traditional housekeeping gene families – actin, tubulin and ubiquitin. Actin is a fundamental cytoskeletal component that is essential to nearly all eukaryotic cells (An et al., 1996). Ubiquitin, which was present in all eukaryotic species examined, directs proteins to compartments in the cell, including the proteasome that destroys and recycles proteins (Callis et al., 1995). The tubulin proteins, including α-tubulin and β-tubulin, make up microtubules in plant growth and development (Chuong et al., 2004). We obtained the candidate reference genes by screening actin, tubulin and ubiquitin genes from our rose EST libraries. The aim was to investigate and validate the internal inference genes for both RT-PCR and qRT-PCR analysis in rose flower (*R. hybrida*) under postharvest stress conditions and during flower opening and senescence.

2. Materials and methods

2.1. Plant materials and initial treatment

Cut rose flowers (*R. hybrida* 'Samantha') were harvested from a local commercial greenhouse and placed immediately in water. Flower-opening stages were defined as described by Wang et al. (2004) and Ma et al. (2005). The flowers were delivered to the laboratory within 1 h of harvesting. Their stems were recut to 25 cm under water and the flowers held in deionized water until needed in a climate-controlled room at 25 °C, 40–50% relative humidity, and a continuous light intensity of 140 μmol m^{-2} s^{-1}.

2.2. Postharvest stress treatments

The flowers were harvested at stage 2 for dehydration, ethylene, ABA, GA_3 and wounding treatments. For dehydration, the flowers were placed horizontally on test-beds and exposed to air for 6, 12, 18 and 24 h. Following 24 h dehydration, the flowers were placed in water for 0.5, 1, 3 and 6 h; to aid rehydration, their stems were recut 1 cm from the base of the stem under water to benefit water uptake and transportation to the flower through the stem. For the acid treatments, the flowers were placed in 50 μmol l^{-1} ABA or 60 μmol l^{-1} GA_3 for 3, 6, 12, 18 and 24 h. For the wounding treatment, the petals were penetrated five to seven times with a needle and sampled after 3, 6, 12, 18 and 24 h penetration. All control flowers were placed in de-ionized water. For exogenous ethylene treatment, 10 ppm ethylene was used, as in a previous study (Ma et al., 2006). The flowers were sealed in a 64 l chamber with ethylene, and, as the control, in regular air for 1, 6, 12, 18 and 24 h. One mol l^{-1} NaOH was put into the chamber to prevent the accumulation of CO_2. For the purpose of RNA isolation, the outermost petals of the flowers were collected and frozen to −80 °C. In order to detect the floral organs during flower opening and senescence, the flowers were harvested at stages 1–6, and the petals, sepals, stamens, gynoecia and receptacles of the flowers were separately taken, sampled and frozen to −80 °C. Individual cut flower was treated as a biological repeat. Three biological repeats were sampled in each analysis.

2.3. Total RNA isolation and cDNA synthesis

Frozen samples from −80 °C were ground in liquid nitrogen using a mortar and pestle. The total RNA of the sepals, petals, stamens and receptacles was extracted using the hot borate method, as described by Ma et al. (2005), and the total RNA of the gynoecia was extracted using the hot phenol method, as described by Xue et al. (2008). Then, the RNA was treated with DNase I digestion (Takara, Japan) to eliminate potential DNA contamination. The integrity of the RNA samples was assessed by gel electrophoresis on 1.5% agarose. One microgram of total RNA was reverse-transcribed into the first strand of cDNA using oligo-dT primers and M-MLV reverse transcriptase.

2.4. Semi-quantitative RT-PCR

Specific primers were designed for RT-PCR analysis using Primer 5 software (listed in Supplemental Table S1). RT-PCR reactions were carried out in a total volume of 25 μl liquid containing: 2.5 μl of 10× buffer, 2 μl of dNTP, 1 μl (10 μmol) of each primer, 1 μl of cDNA template (10-fold diluted from cDNA), 0.5 μl Taq DNA polymerase and 17 μl of sterile distilled water. Thermal conditions were 94 °C for 4 min (denaturation) followed by cycles at 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s. All amplicons were set on 1.5% agarose gel electrophoresis (1/20 EB was included).

2.5. Quantitative real-time RT-PCR and data analysis

Specific primers for qRT-PCR were designed and custom-ordered from a commercial supplier (Sangon, Shanghai, China). Before qRT-PCR, each primer pair was tested via RT-PCR to check for size specificity of the amplicon by electrophoresis on 1.5% agarose gel (1/20 EB was included). qRT-PCR was carried out in 96-well plates with an ABI 7500 Real-Time PCR System and 7500 System Software (Applied Biosystems, Alameda, CA) using an SYBR Green-based PCR assay. qRT-PCR reactions were carried out in a total volume of 20 μl liquid containing: 10 μl 2× SYBR Green PCR mix (KAPA), 2 μl cDNA template (10-fold diluted), 0.4 μl (10 μmol) of each primer, 0.4 μl Rox, and

6.8 μl of sterile distilled water. The thermal conditions were 95 °C for 10 min (denaturation), followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s. Each qRT-PCR analysis was performed in triplicate to increase reliability. The expression stability of candidate reference genes was evaluated by two different statistical packages: geNorm, as described by Vandesompele et al. (2002), and NormFinder, as described by Andersen et al. (2004).

3. Results

3.1. Identification of rose candidate reference genes

In order to identify suitable rose reference genes, the unigenes of traditional housekeeping genes – including actin, tubulin and ubiquitin family protein genes – were selected from three rose EST sources: (1) uniESTs from our subtracted cDNA library under dehydration (Dai et al., 2012); (2) ESTs obtained by 454 massive parallel pyrosequencing technology deposited into an NCBI SRA database under accession number SRA045958; and (3) ESTs collected from the public GenBank. To select rose coding sequences, a BLASTN search using *Arabidopsis* candidate reference genes was performed in our rose transcriptome database (http://bioinfo.bti.cornell.edu/cgi-bin/rose_454/index.cgi). Fifteen of the candidate rose reference genes were selected according to their similarity to reference genes identified in *Arabidopsis*. All putative rose homolog sequences showed more than 75% similarity with the *Arabidopsis* genes. Gene symbols, accession numbers, descriptions and functions according to The Arabidopsis Information Resource (TAIR) are listed in supplementary Table S2. There are four unigenes from the actin protein family, designated as *RhACT1*∼*4*; one α-tubulin designated as *RhTUAI*; three β-tubulins designated as *RhTUB1*∼*3*; and seven from the ubiquitin protein family designated as *RhUBI1*∼*7*.

3.2. Expression profiles of reference genes by RT-PCR

In order to identify reliable reference genes to be used in gene expression studies under different experimental conditions, a timed experiment was designed with the following treatments: dehydration; wounding; exogenous hormones such as ethylene; ABA and GA_3. Using RT-PCR, we first investigated the expression profiles of all 15 candidate genes in petals under stress conditions, as well as in different floral organs, including petals, sepals, stamens, gynoecia and receptacles during rose flower opening and senescence. We found that ubiquitin genes showed more stable expression in rose petals under dehydration and subsequent rehydration, and *RhUBI1*, *RhUBI2* and *RhUBI6* were selected as their candidate reference genes for qRT-PCR analysis (Fig. S1A). Most of the actin, tubulin and ubiquitin genes had relatively stable expressions in petals under exogenous hormone and wounding conditions. *RhACT4*, *RhTUB2* and *RhUBI2* were selected as candidate reference genes for qRT-PCR analysis with ethylene treatment (Fig. S1B); *RhACT4*, *RhTUA* and *RhUBI4* with GA_3 treatment (Fig. S1C); *RhACT4*, *RhTUB3* and *RhUBI2* with ABA treatment (Fig. S1D); and *RhACT2*, *RhACT4* and *RhUBI6* with wounding conditions (Fig. S1E), respectively. There were different expression patterns of 15 candidate genes in rose floral organs during flower opening and senescence. *RhACT1*, *RhUBI2* and *RhUBI6* were selected for qRT-PCR analysis in petals; *RhUBI1*, *RhUBI2* and *RhUBI6* in sepals; *RhACT4*, *RhUBI1* and *RhUBI2* in stamens; *RhACT1*, *RhUBI1* and *RhUBI5* in gynoecia; and *RhACT1*, *RhUBI1* and *RhUBI2* in receptacles, respectively (Fig. S1F). The above 11 genes were used for optimal design of qRT-PCR analysis.

3.3. Verification of primer specificity, PCR amplification efficiency and expression profile of reference genes by qRT-PCR analysis

Based on SYBR Green detection, the qRT-PCR analysis method was used to evaluate the expression stabilities of 11 candidate reference genes under different experimental conditions. Firstly, agarose gel electrophoresis revealed that all primer pairs amplified a specific PCR product of an expected size (Fig. S2A). In addition, target amplicons were sequenced to confirm specificity of the PCR products (data not shown). Specific amplifications were also confirmed by the presence of a single peak in the melting curve obtained after 40 cycles of amplification (Fig. S2B). The PCR amplification efficiency for the 11 reference genes varied from 95.7% for *RhACT1* to 104.4% for *RhUBI1*, and correlation coefficients ranged between 0.971 and 0.995 for *RhUBI5* and *RhTUB2*, respectively (Table S3).

The Ct values of the 11 genes in 10 different sample sets of stress conditions or floral organs were used to estimate the expression levels. The highest Ct value was 28.95 (*RhTUB3* in ABA-treated samples), and the lowest was 17.88 (*RhTUB2* in ethylene-treated samples). Most of the Ct values varied between 20 and 25. In addition, we also found that individual reference genes selected for different experimental conditions, e.g. *RhACT4*, showed different Ct values in the tested sample sets (Fig. 1). These results indicate

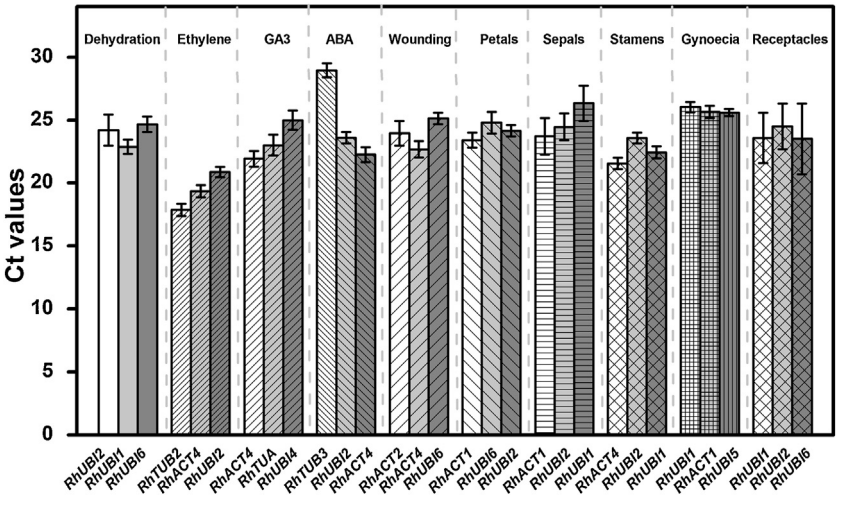

Fig. 1. qRT-PCR CT values for the candidate reference genes in all samples.

Table 1
The expression stability value (*M*) ranking of candidate reference genes calculated by geNorm.

Rank	Dehydration		Ethylene		GA_3		ABA		Wounding	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*RhUBI1*	0.235	*RhACT4*	0.454	*RhACT4*	0.269	*RhACT4*	0.434	*RhACT4*	0.323
2	*RhUBI6*	0.256	*RhTUB2*	0.471	*RhTUA*	0.294	*RhTUB3*	0.449	*RhUBI6*	0.327
3	*RhUBI2*	0.268	*RhUBI2*	0.487	*RhUBI4*	0.308	*RhUBI2*	0.462	*RhACT2*	0.339
Rank	Petal		Sepal		Stamen		Gynoecia		Receptacle	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*RhUBI6*	0.352	*RhUBI2*	0.264	*RhACT4*	0.339	*RhUBI5*	0.253	*RhUBI1*	0.369
2	*RhACT1*	0.381	*RhUBI6*	0.278	*RhUBI2*	0.475	*RhUBI1*	0.262	*RhACT1*	0.445
3	*RhUBI2*	0.409	*RhUBI1*	0.280	*RhUBI1*	0.476	*RhACT1*	0.299	*RhUBI2*	0.474

Note: High *M* values indicate genes with low transcriptional stability, whereas lower *M* values indicate genes with high transcriptional stability.

that none of the 11 reference genes had an invariant expression level. Therefore it is critical to select a reliable reference gene(s) for rose gene expression analysis under postharvest stress conditions or during flower opening and senescence.

3.4. Evaluation of the expression stability of candidate reference genes

The expression stabilities of candidate reference genes were calculated using two different methods: geNorm and NormFinder software. The stability values were determined only in the sample sets of different stress conditions or floral organs.

3.4.1. geNorm analysis

The geNorm algorithm first calculated the expression stability value (*M*) for all three genes in every sample set, and the tested genes were ranked according to their stability values (Table 1). The *M* value is the average pairwise variation of a particular gene with all other tested genes. Using an *M* value below the threshold of 1.5 was generally recommended (Vandesompele et al., 2002), and in our analysis, all genes had an *M* value of less than 1.5, which is considered to be relatively stable. The most stable gene had the lowest *M* value; the least stable gene had the highest *M* value. We found that the *M* values of the 11 genes tested under different conditions were all less than 1.5, showing that the tested genes had stable expression levels. Although three of the candidate genes tested were shown to be stable on RT-PCR analysis, we also found that the difference in stabilities was universal. For example, *RhUBI1* was the most stable gene for dehydration treatment and receptacles during flower opening and senescence, and *RhACT4* exhibited the most stable expression level in petals with exogenous ethylene, GA_3, ABA or wounding treatments, as well as in stamens during flower opening and senescence. In addition, *RhUBI6* in petals, *RhUBI2* in sepals and *RhUBI5* in gynoecia showed the most stable expression levels during flower-opening stages 1–6 (Table 1).

To select a suitable number of reference genes under different experimental conditions, pairwise variation ($Vn/n+1$) was calculated by geNorm analysis. A cutoff value of 0.15 has been widely accepted as the criterion for selecting a suitable number of reference genes, below which the inclusion of additional reference genes is not needed (Vandesompele et al., 2002). However, 0.15 is not an absolute cutoff value, but rather an ideal value. As three candidate genes were analyzed under different experimental conditions, only *V*2/3 pairwise variation values were used in the geNorm analysis. The results showed that the cutoff values in our analysis ranged from 0.09 to 0.159, and only the *V*2/3 value (0.159) with ethylene treatment is slightly higher than the ideal value (0.15) (Fig. 2). This indicated that the two reference genes would be sufficient for normalizing gene expression under our test conditions in rose flowers.

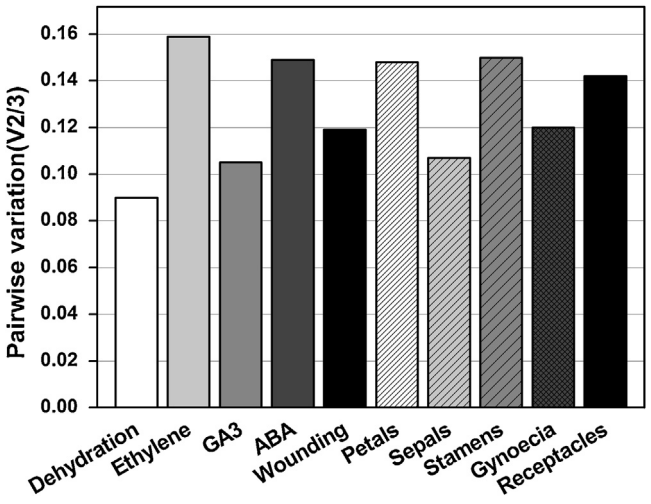

Fig. 2. Pairwise variation (*V*2/3) of the candidate reference genes. As three candidate genes were analyzed under different experimental conditions respectively, only pairwise variation (*V*2/3) was applied by geNorm software.

3.4.2. NormFinder analysis

NormFinder is another algorithm used to identify the optimal reference gene in a group of candidate genes. Different from geNorm, NormFinder takes into account intra- and intergroup variations for normalization factor (NF) calculations, and genes with the lowest NF values are considered to be the most stable (Andersen et al., 2004). This software was used to analyze all stresses and floral organs during flower opening and senescence (Table 2). As with geNorm, NormFinder revealed that *RhUBI1* was the stable gene with dehydration treatment and in receptacles; *RhACT4* was the stable gene with exogenous GA_3 and ABA treatments, wounding and stamens; *RhUBI6* was the stable gene in petals; and *RhUBI2* was the stable gene in sepals. However, NormFinder ranked *RhTUB2* with ethylene and *RhACT1* in gynoecia as their most stable genes, which differed from the results calculated using geNorm. The ranking among the three candidate genes was not consistent between the two algorithms in most of the sample sets. These differences were expected, given that the programs are based on distinct statistical algorithms.

4. Discussion

Gene expression analysis in different tissues and developmental stages and in response to different stress conditions is a major aspect of the gene functional identification. Compared with conventional methods of transcript analysis, qRT-PCR has become the most reliable method of choice for gene expression analysis

Table 2
The expression stability value ranking of candidate reference genes calculated by NormFinder.

Rank	Dehydration		Ethylene		GA_3		ABA		Wounding	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*RhUBI1*	0.083	*RhTUB2*	0.108	*RhACT4*	0.072	*RhACT4*	0.128	*RhACT4*	0.117
2	*RhUBI2*	0.234	*RhACT4*	0.194	*RhTUA*	0.17	*RhUBI2*	0.247	*RhUBI6*	0.158
3	*RhUBI6*	0.308	*RhUBI2*	0.227	*RhUBI4*	0.18	*RhTUB3*	0.254	*RhACT2*	0.225
Rank	Petal		Sepal		Stamen		Gynoecia		Receptacle	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*RhUBI6*	0.111	*RhUBI2*	0.083	*RhACT4*	0.17	*RhACT1*	0.233	*RhUBI1*	0.114
2	*RhACT1*	0.197	*RhUBI1*	0.132	*RhUBI2*	0.214	*RhUBI1*	0.504	*RhUBI2*	0.304
3	*RhUBI2*	0.264	*RhUBI6*	0.135	*RhUBI1*	0.273	*RhUBI5*	0.858	*RhACT1*	0.437

Note: High stability values indicate genes with low transcriptional stability, whereas lower stability values indicate genes with high transcriptional stability.

(Bustin, 2000, 2002). Therefore, qRT-PCR is accepted as the preferred method as it has many distinct characteristics, such as a large dynamic range, tremendous sensitivity, high sequence specificity, and no postamplification processing, and it is responsive to increased sample throughput (Wan et al., 2010). Furthermore, it has been widely used for measuring the expression of genes of interest, validating microarray experiments, and monitoring biomarkers (Chuaqui et al., 2002; Czechowski et al., 2005; Die et al., 2010). However, in spite of these advantages, conventional Northern blotting and RT-PCR are still used for gene expression analysis in much research (VanGuilder et al., 2008).

Regardless of the technique used for gene expression analysis, data normalization is crucial and necessary to obtain reliable gene expression measurements. Different normalization strategies have been proposed (Huggett et al., 2005) and, among them, the use of endogenous, unregulated reference gene transcripts is the most common method (Vandesompele et al., 2009; VanGuilder et al., 2008). Until now, many commonly used reference genes, such as *ACT*, *UBI*, *GAPDH*, *EF1a*, *CYP* and *TUA* have been evaluated for stable expression under specific conditions in many plant species (Czechowski et al., 2005; Exposito-Rodriguez et al., 2008; Jain et al., 2006; Le et al., 2012; Maroufi et al., 2010; Podevin et al., 2012). A similar study was undertaken to identify reference genes for data normalization in different rose tissues under various stress conditions (Klie and Debener, 2011). Common to most of these studies is the aim of selecting and validating a single reference gene from different housekeeping gene families (e.g. *ACT*, *EF1a*, *GAPDH*, *TUA*, *TUB* and *UbI*), with less focus on the expression differences between their homologous genes.

In an earlier study, *PP2A*, *SAND* and *UBC* were identified as suitable reference genes, which, in different combinations, may be used for normalization in qRT-PCR analyses of different rose tissues and stress treatments (Klie and Debener, 2011). However, less attention has been paid to the difference in gene family members, and no special consideration has been given to postharvest factors in the set of test samples. In this study, we preferred to use a set of samples taken from cut rose flowers under abiotic or biotic stresses. We focused on, and collected, the unigenes from three classical housekeeping gene families – actin, tubulin and ubiquitin – and validated them as reference genes for RT-PCR and qRT-PCR analysis. Firstly, we investigated the expression profiles of 15 rose candidate reference genes by RT-PCR under different conditions. Different genes showed different levels of expression stability under different treatments (Fig. S1). The traditional reference genes are not always stably expressed when tested in other species or in a wider range of experimental treatments (Artico et al., 2010; Exposito-Rodriguez et al., 2008; Hu et al., 2009; Mukesh et al., 2006). Based on RT-PCR analysis, we chose three candidate genes with relatively stable expression for further analysis by qRT-PCR under each experimental condition, and there were 11 candidate genes included in all sample sets (Table S3).

As most of the tested candidate reference genes belonged to large gene families, it was necessary to use specific primers (Chen et al., 2011). Although the sequence of rose genome is limited (Debener et al., 2009), we confirmed the specificity of the qRT-PCR primer pairs by agarose gel electrophoresis and melting curve analysis (Fig. S2) and by sequencing of the amplicons. The PCR amplification efficiency was also estimated (Table S3).

The algorithms geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) have been widely used to select the best reference gene for a defined set of biological samples (Cassan-Wang et al., 2012; Czechowski et al., 2005; Mukesh et al., 2006; Yi et al., 2012). Here, we applied geNorm and NormFinder to minimize bias in the quantification of gene expression in cut roses. GeNorm software showed that the stability of the various candidate reference genes varied considerably across the sets of samples (Fig. 1 and Table 1). NormFinder ranks the reference genes according to their expression stabilities, in which a lower stability value means a higher transcriptional stability of gene (Table 2). The most stable gene revealed by NormFinder was almost identical to that revealed by geNorm, except for experimental subsets with ethylene treatment and gynoecia (Tables 1 and 2). These discrepancies between NormFinder and geNorm were also shown in other studies (Boava et al., 2010; Cruz et al., 2009; Hong et al., 2008; Wan et al., 2010), reflecting differences in the statistical algorithms. In our results, these discrepancies may be partially due to the possibility of gene co-regulation within the same family. As a wrong choice of optimum gene would be led while candidate genes were co-regulated in geNorm algorithms (Vandesompele et al., 2002), we finally preferred to conclude our results based on NormFinder analysis. Our results also showed that these candidate genes could be considered to be rather stable as they meet the requirements of both algorithms (Andersen et al., 2004; Vandesompele et al., 2002).

5. Conclusions

In summary, we obtained the expression profiles – via RT-PCR analysis – of 15 candidate reference genes from actin, tubulin and ubiquitin families in rose flower petals under dehydration, ethylene, ABA, GA_3 and wounding treatments. We also obtained the expression profiles of 15 candidate reference genes in different floral organs during flower opening and senescence by RT-PCR analysis. In addition, we tested the expression stabilities of selected candidate reference genes in different sample sets by qRT-PCR analysis. Finally, we proposed the optimal reference genes of rose flowers under different postharvest conditions, e.g. *RhUBI1* for

dehydration treatment and receptacles, and *RhACT4* for GA_3/ABA treatments, wounding and stamens.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scienta.2013.04.019.

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