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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.

1. Introduction

Gene expression analysis has provided crucial information in our understanding of the function of candidate genes, which are 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 gene expression measurements. Different normalization strategies have been proposed (Huggett et 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