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Research article

Identification and validation of reference genes for quantitative real-time PCR studies in *Hedera helix* L.

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ABSTRACT

Reference gene evaluation and selection are necessary steps in gene expression analysis, especially in new plant varieties, through reverse transcription quantitative real-time PCR (RT-qPCR). *Hedera helix* L. is an important traditional medicinal plant recorded in European Pharmacopoeia. Research on gene expression in *H. helix* has not been widely explored, and no RT-qPCR studies have been reported. Thus, it is important and necessary to identify and validate suitable reference genes to for normalizing RT-qPCR results. In our study, 14 candidate protein-coding reference genes were selected. Their expression stability in five tissues (root, stem, leaf, petiole and shoot tip) and under seven abiotic stress conditions (cold, heat, drought, salinity, UV-C irradiation, abscisic acid and methyl jasmonate) were evaluated using geNorm and NormFinder. This study is the first to evaluate the stability of reference genes in *H. helix*. The results show that different reference genes should be chosen for normalization on the basis of various experimental conditions. *F-box* was more stable than the other selected genes under all analysis conditions except ABA treatment; 40S was the most stable reference gene under ABA treatment; in contrast, *EXP* and *UBQ* were the most unstable reference genes. The expressions of *HhSE* and *Hhβ-AS*, which are two genes related to the biosynthetic pathway of triterpenoid saponins, were also examined for reference genes in different tissues and under various cold stress conditions. The validation results confirmed the applicability and accuracy of reference genes. Additionally, this study provides a basis for the accurate and widespread use of RT-qPCR in selecting genes from the genome of *H. helix*.

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1. Introduction

Hedera helix L., a perennial evergreen climbing plants in the family Araliaceae, originated from Europe and is now cultivated worldwide. *H. helix* is often used as an ornamental plant for indoor and vertical gardening because of its evergreen colour, various leaf shapes and strong climbing ability. However, its role as a traditional medicinal plant is often disregarded (Landgrebe et al., 1999; Lutsenko et al., 2010). The fresh leaves and stems of *H. helix* are used to treat cough, asthma, bronchitis and other respiratory diseases (Hofmann et al., 2003; Lutsenko et al., 2010; Cwientzek et al., 2011). Its medicinal value and effectiveness have been accepted by several European countries; thus, this species is recorded in the European Pharmacopoeia as a herbal medicine (European Pharmacopoeia 7.0, 2010). The compounds extracted from this species have also been developed into tablet and syrup formulations (Khdair et al., 2010; Stauss-Grabo et al., 2011). As a medicinal plant, *H. helix* mainly contains triterpenoid saponins, which include hederacoside C, a-hederin, hederacoside B and hederacoside D (Ilhami et al., 2004; Gepdiremen et al., 2005). Although the pharmacodynamics of *H. helix* have been extensively investigated (Fazio et al., 2009; Mendel et al., 2011, 2013; Holzinger and Chenot, 2011), the biosynthesis of triterpenoid saponins in *H. helix* has yet to be reported. Thus far, molecular biology methods have been used to examine functional genes in medicinal plants (Yuan et al., 2008). Biological approaches primarily intend to identify the biosynthetic pathway of active ingredients in medicinal plants and to determine the functional genes and their expression patterns in these pathways; by using biological approaches, researchers can elucidate the regulatory mechanisms of these genes and examine genome diversity (Colebatch et al., 2002).

Reverse transcription quantitative real-time PCR (RT-qPCR) is widely utilized to analyse gene relative expression levels under

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different experimental treatments. This approach provides several advantages, including reproducibility, high sensitivity, accuracy and rapidness; however, its results are affected by the RNA quality, the expression effect of target genes and other factors that contribute to non-uniform test results (Bustin, 2002; Gachon et al., 2004; Derveaux et al., 2010). To obtain the true differences in expressions of target genes by RT-qPCR, we should select stably expressed reference genes for standard correction (Chervoneva et al., 2010). However, the ideal stably expressed reference genes under certain conditions have yet to be reported. The stability of reference genes can be changed using different samples, organs, developmental stages and physiological conditions (Bustin, 2009; Kozera and Rapacz, 2013). In lettuce, *TUB* and *EIF4A1* are the most stable protein-coding reference genes under abscisic acid (ABA) treatment; *EIF2A* and *TIP41* are the most stable genes under drought stress (Borowski et al., 2014). In developmental studies of grapevine berry, *EF1-α* and *SAND* were identified as the most stable reference genes (Reid et al., 2006). In grapevine leaf stress treatment, *EF1-α*, *CYP* and *UBC* are the highest scoring genes (Borges et al., 2014). Thus, we must select suitable and stable reference genes based on different experimental conditions and avoid the random use of one or more reference genes to obtain reliable real-time PCR results. Several medicinal plants, such as *Panax ginseng* (Liu et al., 2014a), *Panax notoginseng* (Wu et al., 2015), *Catharanthus roseus* (Pollier et al., 2014) and *Atropa belladonna* (Li et al., 2014), have been investigated; nevertheless, the selection and estimation of reference genes in *H. helix* remains unperformed. In molecular studies on *H. helix*, a multifactorial analysis is an essential prerequisite to evaluate the stability of reference genes.

In our study, 14 candidate reference genes (*ACT*, *GAPDH*, *18S*, *40S*, *UBQ*, *TUA*, *TUB*, *EF-1α*, *TIP41*, *EXP*, *CYP*, *F-box*, *PGK* and *PP2A*) were selected based on our previous work to identify the most suitable reference genes for normalization of RT-qPCR data obtained from different *H. helix* tissues exposed to various abiotic stresses, including cold, heat, drought, salinity, UV-B, methyl jasmonate (MeJA) and abscisic acid (ABA). The expression stability of these reference genes was analysed using geNorm and NormFinder. The expression of two target genes, namely, *HhSE* (Han et al., 2010; Luo et al., 2011; Liu et al., 2014b; Ye et al., 2014) and *Hhβ-AS* (Kushiro et al., 1998; Haralampidis et al., 2002), which are related to the biosynthesis of triterpenoid saponins in *H. helix*, was also examined to verify the reliability of the selected reference genes. Our data provide a reliable set of reference genes suitable for RT-PCR analysis in *H. helix* under different experimental conditions.

2. Materials and methods

2.1. Plant materials and treatments

H. helix plants were cut in the greenhouse at the National Center for Citrus Improvement at Hunan Agricultural University, Hunan Province, China, on April 11, 2014. The wood cuttings were collected from the Hunan Research Institute of Vine Plants.

One-year-old *H. helix* plant cuttings were used for the greenhouse experiment with completely randomized design and three biological replications. Each biological replicate consisted of 5–8 plants. For different tissue samples, fresh roots, stems, leaves, petiole and shoot tips were harvested, immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction. In all treatments under abiotic stresses, the plants were grown in growth chambers (Life apparatus, Ningbo, China) with a 16/8-h photoperiod, PAR of 300 $\mu mol/m^2/s$ and relative humidity of 60%. Leaf tissues were harvested after treatments and frozen in liquid nitrogen and immediately stored at −80 °C for RNA extraction. For the cold stress treatment, plants were placed at 4 °C for 0, 5, 15 and 30 days. For the heat stress treatment, plants were placed at 40 °C for 2 days. For the drought stress treatment, plants were not watered for 20 days; at the end of this treatment, the measured soil cultivation water content was 5%. For the salinity stress treatment, plants were irrigated with 100 mM NaCl for 2 days. For the UV irradiation treatment, plants were exposed to UV-C radiation (Philips TUV 30 W, 92 $\mu W/cm^2$ at 253 nm) at a distance of 15 cm from the source for 15 min and were then incubated in the dark for 2 days (Borges et al., 2014). For ABA treatment, plants were sprayed with 100 μM ABA for 0, 12, 24 and 48 h. For MeJA treatment, plants were sprayed with 100 μM MeJA for 0, 12, 24 and 48 h.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from all prepared samples with an RNAprep Pure Kit (Polysaccharides & Polyphenolics-rich; Tiangen, Beijing, China) and treated with RNase-free DNase I according to the manufacturer's instructions. The RNA concentration and purity were determined with a Nano Photometer P-Class instrument (Implen, Munich, Germany). The RNA extract had a 260/280 ratio between 1.9 and 2.1 and a 260/230 ratio of approximately 2.0. The RNA integrity was also checked on 1% agarose gels. Total RNA (1.0 μg) was used for reverse transcription with a FastQuant RT Kit (Tiangen, Beijing, China) in a 20 μL reaction volume according to the manufacturer's instructions.

2.3. Primer design and RT-qPCR conditions

The sequences of all the 14 candidate reference genes, including traditional and novel protein-coding reference genes, were obtained from our transcriptome database as constructed by the Illumina Hiseq™ 2500 platform (1Gene, Hangzhou, China). Specific primer pairs were designed with the Beacon Designer 8 software according to the following parameters: primer sequences of 18–24 nucleotides, amplicon length of 75–150 bp, melting temperature (T_m) of 55–60 °C and GC content of 40%–60%. All primer pairs were synthesized using a commercial supplier (Sangon, Shanghai, China) and tested by regular PCR. The products were analysed by electrophoresis on 1.0% agarose gels before RT-qPCR. In addition, the amplification efficiency (E) and correlation coefficient (R^2) were calculated by a standard curve with a 5-fold serial dilution of mixed cDNA (1 μg/μL) (Bustin, 2009). The primer sequences, GeneBank accession numbers, amplicon length, T_m, GC content, E and R^2 of the 14 candidate reference genes are listed in Table 1.

RT-qPCR was performed in 96-well plates in a Bio-Rad CFX96 real-time PCR system (Bio-Rad, CA, USA) with a SYBR Green-based PCR assay. The final volume for each reaction was 20 μL with the following components: 2 μL diluted cDNA template (1 μg/μL), 10 μL SYBR Green Mix (Bio-Rad, CA, USA), 2.5 μL forward primer (2.5 μM), 2.5 μL reverse primer (2.5 μM) and 3 μL ddH_2O. The reaction was conducted under the following conditions: 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 56 °C for 30 s. The melting curve was obtained by heating the amplicon from 65 °C to 95 °C with increments of 0.5 °C in 5 s. Each RT-qPCR analysis was performed with three technical replicates.

2.4. Reference gene expression stability determination and statistical analysis

The two most widely used statistical software programs, geNorm and NormFinder, were used to calculate and evaluate the stability of the 14 candidate reference genes under different experimental conditions. First, raw RT-qPCR data were obtained from the CFX manager (Bio-Rad) and exported into Excel

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