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Abiotic stress and tissue-specific reference genes for quantitative reverse transcription PCR analysis in Korean native watermelons, *Citrullus lanatus* ‘Black-King’ and ‘Speed-Plus-Honey’

Soo Jin Kim^{1,2} · Il Lae Jung² · Hye-Eun Lee³ ·
Ji-Hoon Lee¹

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Abstract A wide variety of research on watermelon has been conducted, and such studies have been motivated by the published genome sequence database of watermelon. Screening of proper reference genes is the primary step for normalization in gene expression analysis. Based on previous studies conducted on *Arabidopsis* and cucumber, we selected eight candidate reference genes of *CI*ACT, *CIEF1*α, *CIGAPDH*, *CI*IDH, *CILUG*, *CI*PTB, *CI*UBC2, and *CI*18S rRNA, respectively, encoding β-Actin, elongation factor 1-α, glyceraldehyde-3-phosphate-dehydrogenase, NADP-isocitrate dehydrogenase, leunig, polypyrimidine tract-binding protein1, ubiquitin-conjugating enzyme E2, and 18S ribosomal RNA from watermelon (*Citrullus lanatus*). The expression levels of these eight genes were evaluated by RT-qPCR under plant hormone-treatment (100 μM ABA) and abiotic stresses such as drought, cold (4 °C), and high salt concentration (250 mM NaCl). The expression patterns of these eight genes were further compared across different types of watermelon tissues such as flower, leaf,

tendril, stem, root, and whole seedling. Our results showed that expressions of *CI*ACT and *CIEF1*α, respectively in the Korean native watermelon cultivars *Citrullus lanatus* ‘Black-King’ and ‘Speed-Plus-Honey’ were least affected by the environmental stresses regardless of tissue types. Here, we suggest two ideal reference genes for watermelon RT-qPCR-based gene expression study.

Keywords Loading control gene · Reference gene · RT-qPCR · Watermelon

Introduction

Watermelon (*Citrullus lanatus*) is one of the most consumed horticultural cucurbitaceous crops and occupies the third place after cucumber and melon, of which the whole genome sequence has been released. Genome sequence and transcriptome data would provide much better opportunities than before for conducting molecular biological studies on crops including watermelon (<http://cucurbitgenomics.org>) [1, 2].

Quantitative reverse transcription PCR (RT-qPCR) has become one of the standard experimental methods for studying gene transcription owing to its accuracy and specificity [3]. In molecular biology, gene expression analysis is broadly performed for multiple experimental purposes including identification of novel gene functions. The results of RT-qPCR depend on transcript normalization of the selected reference genes, which allow regulation of potential variations, caused by exposure to the same conditions as target genes. Therefore, chosen reference genes should be inspected for minimal variability in relative gene expression levels, compared to the experimental samples in advance to the RT-qPCR analysis [4].

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✉ Ji-Hoon Lee
jhlee2@jbnu.ac.kr

- ¹ Departments of Bioenvironmental Chemistry, Chonbuk National University, Jeonju 54896, Republic of Korea
- ² Department of Radiation Biology, Environmental Radiation Research Group, Korea Atomic Energy Research Institute, Daejeon 34057, Republic of Korea
- ³ Vegetable Crop Research Division, Horticultural Department, National Institute of Horticultural and Herbal Science (NIHHS), Rural Development Administration (RDA), 100, Nongsaengmyeong-ro, Iseo-myeon, Wanju-gun, Jeollabuk-do, Republic of Korea

Several suitable reference genes have been identified for some plants including *Arabidopsis* [5]. However, there is no universal reference gene, because the transcription levels of reference genes vary among tissues under different experimental conditions [6]. Moreover, the use of inappropriate reference gene influences the analysis and interpretation of transcription patterns of target genes, resulting in misunderstanding of the functions of the target genes [7, 8]. Therefore, selection of appropriate reference genes is essential for producing correct and reliable results in RT-qPCR experiment.

Stable reference genes in watermelon have not been reported with respect to specific experimental conditions such as drought, cold, and salt stresses. In this research, eight candidate reference genes previously described in watermelons, including of *CICT*, *CIEF1 α* , *CIGAPDH*, *CIIDH*, *CILUG*, *CIPTB*, *CIUBC2*, and *CI18S*rRNA, respectively, encoding β -Actin, elongation factor 1- α , glyceraldehyde-3-phosphate-dehydrogenase, NADP-isocitrate dehydrogenase, leunig, polypyrimidine tract-binding protein1, ubiquitin-conjugating enzyme E2, and 18S ribosomal RNA were chosen, and their transcripts were quantified by RT-qPCR in watermelon tissues under a broad spectrum of stress conditions [9].

Materials and methods

Plant materials

Seeds of watermelon cultivars *Citrullus lanatus*. ‘Black-King’ and ‘Speed-Plus-Honey’ were obtained from the Nongwoobio Seed Company (Suwon, Republic of Korea). To study the effects of abiotic stresses on seedling growth, Black-King (BK) and Speed-Plus-Honey (SPH) seeds (commercial F1 hybrid) were sterilized with 30% sodium hypochlorite (bleach) for 15–20 min, rinsed for 10 times with distilled water, germinated in Murashige and Skoog (MS) medium with 3% sucrose (pH 5.8) and 0.8% phytoagar, and grown in a growth chamber at 25 °C under long-day conditions (16 h light and 8 h dark) [10]. The watermelon plants used for the tissue-specific expression pattern analysis were cultured in a greenhouse [11].

Abiotic stress treatment and sampling

The BK and SPH watermelon seedlings were subjected to drought, cold, salt, and abscisic acid (ABA) treatments. The BK and SPH watermelons were raised in agar plates for 2 weeks. The harvested whole seedlings from the plates were dehydrated on 3MM filter paper (Whatman) at around 25 °C and approximately 60% humidity under dim light. The 2-week-old seedlings were soaked in the MS liquid

media containing 300 mM NaCl for salt stress, or 100 μ M ABA for ABA treatment for 2 h. For cold stress treatment, the watermelon seedlings were transferred to an incubator set at 4 °C for 6 h. Whole seedlings were harvested, promptly soaked in liquid N₂, and stored at – 80 °C until extraction of the total RNA from samples [12, 13].

Selection of candidate reference genes and primer design for qPCR

To identify proper loading control genes for watermelon, eight candidate genes were evaluated. The eight genes were chosen based on previous studies conducted on watermelon and other crops. The selected genes were *CICT*, *CIEF1 α* , *CIGAPDH*, *CIIDH*, *CILUG*, *CIPTB*, *CIUBC2*, and *CI18S*rRNA as described earlier. To obtain orthologous reference genes in watermelon, *Arabidopsis* homologs of the selected eight genes were used for blastn search against watermelon genomes in the Cucurbit Genomics Database. The coding DNA sequences with the best hit were chosen and uploaded to Beacon Designer (http://www.premierbiosoft.com/molecular_beacons/) and Primer3Plus software (<http://primer3plus.com>) for designing primers. The primers of the loading control genes used for RT-qPCR in this study were designed following previously described protocols [9]. Information on the chosen reference genes is listed in Table 1. For more comparable results, the primer pair for 18S rRNA gene, which was previously published, was used in this study [1]. Results on the selected eight genes for loading control and their amplification characters are listed in Tables 1 and 2.

Total RNA extraction, RT-PCR, and RT-qPCR

Total RNA of the watermelon was extracted from drought-, cold-, and salt-stressed and ABA-treated 14-day-old watermelon seedlings using a commercial RNA extraction kit (Intron Biotech., Daejeon, Republic of Korea) according to the protocol. cDNA synthesis and RT-PCR were performed following previously described protocols [14]. Real-time RT-qPCR was performed on a CFX96 Real-time System (Bio-Rad, Hercules, CA, USA), and data of RT-qPCR were analyzed with CFX Manager program (Bio-Rad). PCR reactions were conducted as described previously [13]. The PCR cycling conditions were described previously [12]. The melting curves were recorded after forty cycles to test primer specificity by heating from 55 to 95 °C. Three replicates were conducted for each sample. Negative controls without any cDNA as templates were included. Amplification efficiencies of RT-qPCR for all primer pairs for eight candidates genes were evaluated using the serial tenfold dilutions of the pooled cDNAs (0.5, 5, 50, and 500 ng) [15].

Table 1 Description of watermelon candidate reference genes for qRT-PCR

Genes	Descriptions	IDs	Homologue locus for <i>Arabidopsis</i>	E-value
<i>CIACT</i>	β -actin	<i>Cla007792</i>	<i>At5g09810</i>	0
<i>CIEF1α</i>	Elongation factor 1- α	<i>Cla010539</i>	<i>At1g07940</i>	0
<i>CIGAPDH</i>	Glyceraldehyde-3-phosphate-dehydrogenase	<i>Cla013454</i>	<i>At1g16300</i>	1e–105
<i>CIIDH</i>	NADP-isocitrate dehydrogenase	<i>Cla009135</i>	<i>At1g65930</i>	1e–71
<i>CILUG</i>	Leunig	<i>Cla022288</i>	<i>At4g32551</i>	7e–62
<i>CIPTB</i>	Polypyrimidine tract-binding protein1	<i>Cla004906</i>	<i>At5g53180</i>	5e–92
<i>CIUBC2</i>	Ubiquitin-conjugating enzyme E2	<i>Cla010164</i>	<i>At2g02760</i>	4e–81
<i>CI18SrRNA</i>	18S ribosomal RNA			

Table 2 Information about primer sets of watermelon candidate reference genes for qRT-PCR

Genes	Primer sequences 5'–3'	Product size (bp)	Amplification efficiency (%)	R ²
<i>CIACT</i>	F:CCATGTATGTTGCCATCCAG R:GGATAGCATGGGGTAGAGCA	140	93.4	1.000
<i>CIEF1α</i>	F:GCACGCTCTTCTTGCTTTC R:ACGATTTTCGTCGTACCTTGC	115	89.6	1.000
<i>CIGAPDH</i>	F:CTGGCAGTACTTTGCCAACA R:AGGATTGGAGAGGAGGTCGT	87	97.5	0.999
<i>CIIDH</i>	F:TGGCCTCTTTACCTAAGCACA R:ATATGCCAGCAGCCTCAAAC	124	92.1	1.000
<i>CILUG</i>	F:TTGCTGGTCATTGGATGCTA R:GCCGAAGCAACTAGACCTGA	138	86.8	0.995
<i>CIPTB</i>	F:GGAGCAAACAGAAATCAAGC R:AGCAGGCTCAGAGGAAGATG	133	93.5	0.997
<i>CIUBC2</i>	F:CCAAATAAGCCACCGACAGT R:TCATAGATTGGGCTCCATTG	118	95	0.999
<i>CI18SrRNA</i>	F:AGCCTGAGAAACGGCTACCACATC R:ACCAGACTCGAAGAGCCCGGTAT		52.6	0.999

Results and discussion

Conditions for PCR amplification of the reference genes

From the watermelon genome sequence, homologous genes were obtained by examining sequence similarity hits. All the primers' information used in this study is listed in Tables 1 and 2.

Results of melting curve analysis are shown in Fig. S1 (in Supplemental Material). The presence of a single peak without primer-dimer formation indicates only amplification specificity for each reference gene.

Standard curve result analysis determined the efficiency of the real-time RT-qPCR for each primer pair and was generated by using four serial tenfold dilutions of cDNA, ranging from 52.6% (*CI18SrRNA*) to 97.5% (*CIGAPDH*).

The determination of coefficients (R^2) of the standard curve regression equation varied from 0.995 (*CILUG*) to 1.000 (*CIACT*, *CIEF1 α* , and *CIIDH*) (Table 2). The results implied that specific and efficient RT-qPCR systems were established to quantify the selected reference genes, except *CI18srRNA*.

Gene expression analysis

The mRNA levels of eight reference genes in two cultivars, *Citrullus lanatus* 'Black-King (BK)' and 'Speed-Plus Honey (SPH)' expressed in watermelon seedlings subjected to a broad spectrum of abiotic (drought, cold, and salinity) and ABA hormone stresses, are presented as quantification cycle (C_q) values in Fig. S2. Different transcript abundances were observed for the eight genes. However, the mRNA transcription levels of the genes in

Fig. 1 Expression profiles of watermelon candidate reference genes. The mean C_q values of all the stress experiments in each gene are presented with RSD values for Black-King (A) and Speed-Plus-

Honey (B). The average C_q values per tissue in each gene are also presented with RSD values for Black-King (C) and Speed-Plus-Honey (D)

the two varieties, BK and SPH, were almost the same. *CI18SrRNA*, which had the highest mean C_q value of 30 for both the BK and SPH varieties, had the lowest mRNA expression level among all the candidate genes. The expression levels for *CIActin*, *CIEF1 α* , *CIIDH*, and *CIUBC2* were similar and ranged from 21 to 22 cycles. The expression levels for *CIGAPDH*, *CILUG*, and *CIPTB* were comparable and ranged from 25 to 27 cycles. The average C_q values of *CIActin*, *CIEF1 α* , and *CIUBC2* were within the range of 21 to 22 cycles. Moreover, these three genes, among the eight selected loading control genes, exhibited a constant expression level in all the stressed seedlings. The variability of C_q values in the abiotic stress-treated samples was highest for *CI18SrRNA* and *CIIDH*, whereas *CIActin*, *CIEF1 α* , and *CIUBC2* showed the lowest variations in gene expression (Fig. S2).

To get reliable reference genes, the C_q values of those eight selected reference genes in the abiotic stress-treated samples were evaluated by the standard deviation (SD) and relative standard deviation (RSD) values. In Fig. 1, the RSD values of the eight genes were from 0.93 to 4.54%, which were all in the acceptable range [16]. Especially, the RSD values of *CIActin*, *CIEF1 α* , and *CIUBC2* were less than 1%. Therefore, the *CIActin*, *CIEF1 α* , and *CIUBC2* genes were selected for further analysis.

To elucidate the variability of transcript levels of *CIActin*, *CIEF1 α* , *CIUBC2*, and *CIGAPDH* in the flowers, leaves, tendrils, stems, roots, and whole seedlings of watermelons SPH and BK, RT-qPCR analysis was carried out (Fig. S3). Average C_q values of *CIActin*, *CIEF1 α* , or *CIUBC2* among the different tissues were comparable to each other with minor variations. Average C_q values of *CIGAPDH* ranged from 24 to 28 in tissues of two different

watermelon cultivars which were used in this paper. Therefore, this gene was not considered proper as a tissue-specific reference gene, because the difference of C_q was about 3 to 4. As a result, *CIACT*, *CIEF1 α* , and *CIUBC2* were considered appropriate reference genes for tissue-specific study.

In case of BK watermelon, *CIACT* would be the most proper reference gene when tissue-specific gene expression test would be performed. The gene *CIEF1 α* was the most appropriate as the reference gene in tissue-specific transcription level test of SPH watermelon. These two genes were selected on the basis of the least SD and RSD values. The SD and RSD values of BK watermelon were 0.49% and 2.35%, respectively. The SD and RSD values of SPH watermelon were 0.60% and 2.93%, respectively (Fig. 1). Therefore, we suggest *CIACT* of BK and *CIEF1 α* of SPH as reference genes for the tissue-specific gene expression experiment. Considering the C_q , SD, and RSD values of the diverse abiotic stresses and tissues, it seems that *CIACT* gene is suitable as a reference gene for BK watermelon and *CIEF1 α* gene for SPH watermelon.

In cucumbers, one of the cucurbitaceous crops, the genes of *EF1 α* , *F-box*, and *CAC* (*Clathrin Adaptor Complex Subunit*) have been reported to be stable genes under diverse abiotic stresses and growth regulatory treatments [17]. In another study on cucumbers, the genes *EF1 α* , *UBI-ep* (*Ubiquitin like protein*), and *TUA* (α -*Tubulin*) were also shown to be most stable [18]. For melons, genes encoding ribosomal protein L2 (*RPL2*), actin (*ACT*), and cyclophilin (*Cyp*) were the three best loading control genes, when melon stems were infected with *Fusarium* wilt [19].

In conclusion, among the eight watermelon genes studied here, *CIACT* and *CIEF1 α* were found to be suitable reference genes, respectively, for BK and SPH watermelons in order to study normalization of gene expression in watermelon under many kinds of environmental stresses and diverse tissues. The optimal reference genes selected in watermelon were different from the reference genes in cucumber and melon, even though these three are all cucurbitaceous crops. Moreover, watermelon had different reference genes that were suitable for different varieties. Therefore, we suggest that *CIACT* and *CIEF1 α* genes would be suitable as the reference genes for BK and SPH watermelons, respectively.

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