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Selection of reliable reference genes for gene expression studies in *Botrytis cinerea*

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ABSTRACT

Botrytis cinerea is an important plant pathogen causing grey mold disease in a wide range of plant species. The aim of this study was to identify reliable reference genes that can be used for the analysis of relative gene expression in *B. cinerea* with quantitative real-time reverse transcription PCR (qRT-PCR). Six commonly used housekeeping genes including actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ubiquitin (*UBQ*), ubiquitin-conjugating enzyme (*UCE*), α-tubulin (*α-TUB*) and β-tubulin (*β-TUB*) were selected to test their expression stabilities in *B. cinerea* treated with different concentration of oxalic acid (1, 5 and 10 mM) and confronted with antagonistic *Trichoderma afroharzianum*. Four *in silico* algorithms (geNorm, BestKeeper, NormFinder and Comparative ΔCt) were applied to evaluate the expression stabilities of these genes, and the *UBQ* gene was identified as the most stably expressed. It was used to normalize the expression levels of three genes related to oxalic acid production (*NADPH*, *VEL1* and *OAH*) when *B. cinerea* was challenged by *T. afroharzianum*. The results of this study are useful for gene expression analysis in *B. cinerea*.

1. Introduction

Botrytis cinerea is an airborne plant pathogen with a necrotrophic lifestyle attacking over 200 crop hosts (including field and row crops, fruit, vegetable and flowers) and causing destructive and economically important plant diseases worldwide. The infection of host plants is mediated by a range of cell wall-degrading enzymes, toxins and other low-molecular-weight compounds such as oxalic acid (OA) (Williamson et al., 2007). These compounds may play different roles during the infection process (Nakajima and Akutsu, 2014). OA enhances polygalacturonase activity (Bateman and Beer, 1965), inhibits plant defense responses (Cessna et al., 2000; Favaron et al., 2004; Marciano et al., 1983), mediates pH signaling (Rollins, 2003), and induces plant programmed cell death (Kim et al., 2008). So OA is an important virulence factor in pathogenesis of *B. cinerea*.

Degrading OA was reported to be a useful approach as a disease control (Paramasivan et al., 2013), and OA degradation is one of the desired activities of biological control agents. We found that *Trichoderma afroharzianum* strain LTR-2 could eliminate OA in liquid culture containing 20 mM OA (Wu et al., 2016). Oxalate decarboxylase can catalyze the decomposition of oxalate to formate and CO_2 (Anand et al., 2002), and its coding gene *OXDC* in strain LTR-2 was found to be up-regulated significantly under OA stress (Lyu et al., 2017). But the expression changes of the genes related to OA production in *Botrytis cinerea* during the interaction with *T. afroharzianum* were still unknown.

The quantitative real-time reverse transcription PCR (qRT-PCR) method is a good tool for quantifying target gene expression in specific conditions or evaluating gene expression changes, which has high sensitivity and specificity. Reliable quantification with this method depends on the use of stably expressed endogenous genes as reference genes (Busk, 2014; Hellemans and Vandesompele, 2014; Bustin et al., 2009; VanGuilder et al., 2008). A suitable reference gene is necessary for quantifying gene expression and the reference gene should be stably expressed under specific experimental treatments. Although qRT-PCR has been widely used in quantifying gene expression in *B. cinerea* (Malmierca et al., 2016; Zhang et al., 2014; Zhang et al., 2016a, 2016b), the selection of reference genes has not been systematically evaluated so far. In order to screen the most reliable reference gene in *B. cinerea*, six housekeeping genes including actin gene (*ACT*), glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*), ubiquitin gene

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(*UBQ*), ubiquitin-conjugating enzyme gene (*UCE*), α-tubulin gene (*α-TUB*) and β-tubulin gene (*β-TUB*) (Shivhare and Lata, 2016; Sun et al., 2015) were selected as candidate reference genes and their expression stabilities were tested. Four *in silico* algorithms including BestKeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and Comparative ΔCt (Silver et al., 2006) were used to evaluate their expression stability. This work was undertaken to identify the most reliable reference gene in *B. cinerea*.

2. Materials and methods

2.1. Strains

B. cinerea strain S31 was originally isolated from moldy cucumber (*Cucumis sativus* L.) in a greenhouse in Tai'an, Shandong, China. *T. afroharzianum* strain LTR-2 was originally isolated from a soil sample in Jinan, Shandong Province. Both of the strains were maintained in our lab.

2.2. Experimental conditions

B. cinerea S31 was cultured on the center of PDA (Hope, China) plates (diameter 90 mm), and incubated at 25 °C for 6 days, the same growth condition and assay were performed for *T. afroharzianum* strain LTR-2.

As shown in Table 1, treatments in this experiment included five different groups: mycelial plug (diameter 5 mm) of strain S31 was cultured alone as the treatment S; strain S31 was dual cultured with strain LTR-2 as the treatment S-T; initial concentration of 1, 5 and 10 mM OA were added in the treatment S as S + 1, S + 5 and S + 10, respectively. OA (Bodi, China) stock solution (0.5 M) was sterilized by filtering through the 0.22-μm PES membrane before adding into plates. In all treatments, PDA plates were covered with sterile cellophane in advance, and all plates were incubated at 25°C for 90 h after inoculating *B. cinerea*. *T. afroharzianum* plugs were inoculated 48 h later than *B. cinerea* to make their mycelia just contact at the harvest time. Three replicates were performed for each treatment.

2.3. Amplification of reference genes

Six housekeeping genes that were frequently-used in eukaryotes were selected from *B. cinerea* to screen for stable gene expression *via* the qRT-PCR technique, *i.e.*, *ACT*, *GAPDH*, *UBQ*, *UCE*, *α-TUB*, and *β-TUB*. The sequences of these genes were obtained from the genome database of *B. cinerea* strain B05.10 at GenBank (https://www.ncbi.nlm.nih.gov/genome/494). All primer pairs were designed and evaluated *via* Beacon Designer 7.9 (PREMIER Biosoft, USA) with amplicon sizes ranging from 75 to 150 bp and synthesized by Sangon Biotech (Shanghai, China). The specificity of the primers was verified by conventional PCR, and those primer pairs with a single band having the expected size in a 2% agarose gel electrophoresis were listed in Table 2.

2.4. Total RNA extraction and cDNA synthesis

The mycelial samples collected from each treatment group were immediately snap frozen and ground into a fine powder under liquid nitrogen. Total RNA was extracted with the Fungal RNA Kit (OMEGA, USA) following the manufacturer's instructions. The quantity and quality of RNA samples was checked using a BioSpec-nano spectrophotometer (Shimadzu, Japan). TURBO DNase™ (Thermo, USA) was used to digest DNA contamination. First strand cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo, USA). All cDNA products were diluted 10-fold prior to use.

Table 1
Treatments assays on PDA plate.

Treatment name	Treatment profile
S	*B. cinerea* S31 alone
S-T	*B. cinerea* S31 dual cultured with *T. afroharzianum* LTR-2
S + 1	*B. cinerea* S31 treated with 1 mM OA
S + 5	*B. cinerea* S31 treated with 5 mM OA
S + 10	*B. cinerea* S31 treated with 10 mM OA

Table 2
Candidate reference genes and primers.

Reference genes[a]	Accession number	Primers of positive-sense and anti-sense(5′-3′)	Fragment length (bp)
GAPDH	XM_001555875.1	F-TGCCAAGAAGGTTGTTATC R-TGTAGGTCTCGTTGTTGA	77
β-TUB	XM_001560987.1	F-TGAAGGTATGGACGAGAT R-GCATCCTGGTATTGTTGA	84
ACT	XM_001553318.1	F-TCTGTTATGTTGCTCTTGAT R-GTTCGTATGACTTCTCCAA	78
UCE	XM_001546665.1	F-ATCACCCAAACATCAACT R-CATAGAGCAGATGGACAA	107
UBQ	XM_001556819.1	F-CAAGGTTACCGACAACAATA R-GCATCCATCAACTTCTTCAA	75
α-TUB	XM_001555875.1	F-GTTGGAGTTCTGTGTCTA R-GTGGTCAAGATGGAGTTA	75
NADPH	XM_001558572.1	F-GATCAATATCTGGAATGGT R-CGTCCGAATACAATTACA	120
VEL1	HE977589.1	F-CTCTCAACAACAATCGTA R-GGCTATAAGTAGGTTCCA	146
OAH	XM_001557841.1	F-CTCCAAGAGAAGTCTATT R-CATCATAAGGTGTAACAG	79

[a] *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; *β-TUB*: β-tubulin; *ACT*: actin; *UCE*: ubiquitin-conjugating enzyme; *UBQ*: ubiquitin; *α-TUB*: α-tubulin; *NADPH*: NADPH dehydrogenase; *VEL1*: VELVET1; *OAH*: Oxaloacetic acid acetyl hydrolase.

2.5. qRT-PCR and expression stability of reference genes

The qRT-PCR reactions were carried out on a Bio-Rad CFX96 touch qPCR System (Thermo, USA). Each reaction was performed in a total volume of 15 μL containing 7.5 μL of 2 × SYBR Green PCR Premix HS Taq (Genview, USA), 1 μL of cDNA, 0.75 μL of each primer with final concentration of 500 nM, and 5 μL ddH_2O. The qRT-PCR cycling conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 53 °C for 20 s, and 72 °C for 20 s. qRT-PCR products were verified by subsequent melt curve analysis and electrophoresis. Two technical replicates were included in each PCR run to ensure reproducibility. The expression stabilities of the six reference genes were evaluated using four *in silico* algorithms (BestKeeper, geNorm, NormFinder and Comparative ΔCt). The candidate reference genes were ranked by each algorithm individually. The geometric means of these ranks were used to determine their expression stability, with the lowest value representing the most stably expressed gene while the highest representing the least stably expressed.

2.6. Relative quantification of OA production related genes

Oxaloacetic acid acetyl hydrolase gene (*OAH*) participates in catalyzing the hydrolytic cleavage of oxaloacetate to form acetate and oxalate (Han et al., 2007). NADPH dehydrogenase gene (*NADPH*) (Amselem et al., 2011) and VELVET1 gene (*VEL1*) (Schumacher et al., 2012) are involved in producing the virulence factor OA in *B. cinerea*. These three target genes were selected to study their expressions during the confrontation test of *B. cinerea* with *T. afroharzianum* using the designated reference gene. The primer pairs used for *NADPH*, *VEL1* and *OAH* are designed using the program described above (Table 2). The reaction system was the same as for qRT-PCR of reference genes, and the relative expression levels of target genes were calculated using the