Evaluation of Reference Genes for the Relative Quantification of Apple stem grooving virus and *Apple mosaic virus* in Apple Trees

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Abstract A SYBR Green[®]-based one step RT-qPCR assay was developed for the detection and quantification of *Apple stem grooving virus* (ASGV) and *Apple mosaic virus* (ApMV). The RT-qPCR assay employed seven plant-expressed genes—glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA, ubiquitin, ribosomal protein S19, Rubisco, RNA polymerase subunit II and β -actin—as internal reference housekeeping genes in a relative quantification system in three apple cultivars (i.e. Idared, Champion, Fragrance). The average expression stability (*M*) found by GeNorm software suggest that GAPDH and S19 were the most stable reference genes. We propose employing GAPDH and S19 as housekeeping genes for accurate quantification of ASGV and ApMV in apple leaf samples. The detection limit for both viruses was found around 70 copies of viral genome by one-step RT-qPCR.

Keywords qPCR · ASGV · ApMV · Reference genes

The fluorescence-based quantitative real-time PCR (qPCR) has been used to detect and measure nucleic acids of a wide range of plant viruses with RNA and DNA genome [13, 16, 17]. Two main qPCR based techniques: relative and absolute quantification have emerged from various studies on virus detection and quantification of virus titer. The “absolute quantification” with known standard is the most commonly used for plant virus quantification. On the other hand, relative quantification uses a comparative quantification with internal reference endogenous housekeeping

control genes [1]. The relative quantification determines the changes in RNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. Therefore, relative quantification does not require standards with known concentrations [15]. Relative quantification employing reference internal control genes was used for several plant virus detection and quantification studies [5, 6]. A comparative study showed that the relative quantification assay may be as accurate as absolute quantification when appropriate housekeeping genes are used and normalized with target genes [6, 10]. In this paper we have chosen endogenous housekeeping genes for quantification of viruses in apple trees in a relative quantification system. Two apple tree infecting viruses, *Apple stem grooving virus* (ASGV) and *Apple mosaic virus* (ApMV) are selected. ASGV and ApMV belong to the most frequently emerged pathogens encounters in pome fruit trees. Several molecular-based methods have been elaborated to perform routine detection [7, 8, 11, 12]. However, a highly sensitive real-time based detection and quantification assay for these viruses is missing so far. We described here a SYBER green based relative quantification assay employing appropriate endogenous reference genes as internal control.

A total of 39 samples coming from different apple cultivars grown in vitro culture (i.e. Idared, Champion, Fragrance) healthy (14 plants) and infected (25 plants) have been used to validate the reference genes as well as the target genes. The leaves of plant material were grinded in liquid nitrogen and the total RNA was extracted with the Spectrum Plant Total RNA Kit (Sigma-Aldrich) according the manufacturer’s instruction. The concentration was evaluated in duplicates by nanodrop photometer (Implen GmbH) and 2 μ g of total RNA was processed by DNase I treatment (Ambion, USA) (2U DNase I, 10 $\times$ DNase I buffer in 20 μ L

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final volume) and DNase I was inactivated by inactivation buffer (Ambion, USA). The remaining DNA contamination was checked by PCR for reference genes using following protocol: initial denaturation 94 °C 3 min, followed by 30 cycles of 94 °C 1 min, 60 °C 30 s, 72 °C 1 min and final elongation 72 °C for 3 min. Finally, 5 µL of the PCR product were run in 1.5% agarose gel colored by Sybrgreen (Sigma-Aldrich) and visualized under UV light. Seven reference genes such as GAPDH [3], 18S rRNA [3], S19 [3], Ubiquitin [2], Rubisco [4], RNA polymerase [4] and β -actin [9] as well as for the two viruses ASGV and ApMV [12] were chosen. These internal genes are known as the most stable expression in apple tissues Table 1.

The real-time RT-PCR was performed using 7300 Real-Time PCR System (Applied Biosystems, CA, USA) and the *Power SYBR Green RNA-to-Ct*® 1-step Kit (Applied Biosystems, CA, USA) as simplex PCR but performed in the run. The cycling conditions were: 48 °C 30 min (Reverse-transcription step), 95 °C 10 min (Inactivation of the reverse transcriptase) and 40 cycles of 95 °C 15 s and 60 °C 1 min. A duplicate of no template control (NTC) in every run for each primer pair to test buffers and solutions for DNA contamination and to assess for primer-dimers and combined with a dissociation stage to verify the specificity of each couple of primers and check the non specific amplification.

The impact of the infectious status (healthy or infected) on reference genes expression was statically evaluated. First of all the null hypothesis (Shapiro–Wilk test [18]) was used to determine whether the dataset set is well-modeled by a normal distribution and determine if the *t* test parametric test could be subsequently used to compare the two groups, as the results were not convincing the Mann–Whitney non parametric test was preferred. The impact of the apple cultivars (Idared, Champion, and Fragrance) on the expression of the reference genes was analyzed by the two-way ANOVA statistical test with a Bonferroni posttest. For the detection limit of the apple viruses the one step RT-qPCR was used, 2 µg of RNA produces by in vitro transcription (MEGA-script® T7 Kit-Ambion, Inc.) and corresponding to the target sequence of the respective viruses (ASPV or ApMV) was treated by DNase I (Ambion, USA) to eliminate the

plasmid, DNase I was inactivated by inactivation buffer (Ambion, USA) and 10 times serial dilution was done. The number of virus copies was evaluated according the formula as follow: conversion of microgram of single stranded RNA to picomole was performed considering the average molecular weight of a ribonucleotide (340 Da) and the number of bases of the transcript (Nb). $\text{pmol of ssRNA} = \mu\text{g (of ssRNA)} \times (10^6 \text{ pg/1 } \mu\text{g}) \times (1 \text{ pmol/340 pg}) \times (1/\text{Nb})$. Avogadro constant was used to estimate the number of transcripts (6.023×10^{23} molecules/mol) [14].

First of all, the impact of the infected status was analyzed by Mann–Whitney non parametric and no difference in reference genes expression within infected and healthy plants was observed ($P > 0.05$). Thereafter another analyze was performed to investigate the effect of plant cultivars in the expression of reference genes. The analysis was performed by two-way ANOVA statistical test with a Bonferroni posttest. Finally, the stability of expression of reference genes were evaluated using virus infected and non-infected apple leave tissue by GeNorm tools [20]. The efficiency of the each primer pairs was evaluated according the equation: $E = 10^{(-1/\text{slope})}$ for a serial dilution of RNA. The efficiency ranged between 90.49 and 103.40% for the reference genes and target genes, which is within the suggested values (90–110%) for real-time assays [19]). The average expression stability *M* found by GeNorm point out the GAPDH, S19 as most stable reference genes in our experiment with a *M* value about 0.36. The Fig. 1 presents the pairwise variation *V* between two sequential normalization factors containing an increasing number of genes and reveals that two reference genes are sufficient for correct normalization; cut-off value <0.15 reaches in V2/3 [20]. GeNorm software was used to analyze the stability of tested reference genes and determine the most stable combination and the optimal number for acute and robust results. The sensitivity of the primers for the target genes of ASGV and ApMV was evaluated in one step RT-qPCR system. The qPCR was afterwards performed according to the same conditions described above. All the infected samples tested for the respective viruses were positives; no cross-reaction was observed with ASGV primers as well as ApMV with the others viruses or healthy samples.

Table 1 Efficiencies and Cq values obtained for the references and target genes

	Target genes		References genes	
	ASGV	ApMV	GAPDH	S19
Average Cq values				
Dilution 1/10	22.29 ± 0.06	18.33 ± 0.33	19.98 ± 0.24	20.64 ± 0.24
Dilution 1/100	25.57 ± 0.02	21.20 ± 0.33	23.00 ± 0.27	23.94 ± 0.24
Dilution 1/1,000	29.35 ± 0.09	24.23 ± 0.19	26.49 ± 0.35	27.39 ± 0.31
Dilution 1/10,000	32.94 ± 0.6	28.50 ± 0.6	30.40 ± 0.32	30.30 ± 0.40
Average efficiency	90.49%	98.60%	93.98%	103.40%

Cq values provided in this table are the results of triplicate experiment and given with standards errors

Fig. 1 Pairwise variation V between two sequential normalization factors containing an increasing number of genes. A cut-off value of 0.15 [20] below which the insertion of additional reference genes is not required

Fig. 2 Agarose gel 2% of detection and sensitivity of *Apple stem grooving virus* (A) and *Apple mosaic virus* (B) by one step RT-PCR

We proposed GAPDH and S19 as the two most stably expressed reference genes for the accurate quantification of ASGV and ApMV in apple trees. The one-step RT-qPCR describes here is able to detect both viruses (ASGV, ApMV) up to the dilution 10^{-10} of sample/RNA of apple leaf tissue (Fig. 2). This dilution end point corresponds to ~ 70 copies of viral genome.

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