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Real-time RT-PCR quantitative analysis of plant viruses in stone fruit tissues

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Abstract

Real-time PCR assays aiming at quantifying the level of plant infection by pathogens are becoming more and important. Within microbiology, the application of Real-time PCR has had the biggest impact upon the field of virology. However, Real-time PCR application in fundamental plant virology studies is still lagging behind. The use of relative and absolute quantification is discussed in this study. Also, case studies including Plum pox virus in transgenic plums C5 are presented.

Keywords: Plum pox virus, C5, Real-time PCR, quantitation

Introduction

Real-time PCR with melting curve analysis has already proved to be a simple, rapid, and reliable technique for the detection of plant RNA viruses (Varga and James, 2005). These highly sensitive assays were employed only for virus detection rather than quantification of virus inoculum. Real-time PCR assays aiming at quantifying the level of plant infection by pathogens have been increasing for the last few years (Gachon et al., 2004) in multiple areas of viral pathogenesis. For means of quantifying a virus in tissues, absolute quantification is temporarily, beyond controversy, the preferred way of Real-time PCR. Here, it should be kept in mind that the obtained 'absolute numbers' are always calculated relative to the standard (RNA, cDNA, plasmid DNA, genomic DNA) and largely depend on the accuracy of the used standard (Klein, 2002). For many applications, where there is no need to know the exact number of copies in the sample, a relative quantification using a comparative quantification method ($\Delta\Delta\text{CT}$ -method) is sufficient (Mackay, 2004). To ensure the accuracy of the obtained data, the reaction efficiencies of the two assays either should be similar or a correction factor must be introduced into the calculation (Klein, 2002). Relative quantification in virology was neglected for a long time, even though, when conducted carefully, its data can be at least as precise as the data of absolute quantification. The aim of this study was to compare absolute and relative quantification by Real-time PCR and uniformity of results reached by both methods. For this, a case study monitoring Plum pox virus (PPV) titre in transgenic plum trees C5 was chosen. PPV is the causal agent of sharka, one of the most important diseases affecting commercial stone fruit (Németh, 1986). Six different strains of PPV have been described until now (Candresse et al., 1998; Glasa et al., 2004; James and Varga, 2005; Kerlan and Dunez, 1979; Nemchinov and Hadidi, 1996; Wetzel et al., 1991). Transgenic C5 cv. 'Honey Sweet' is a clone of *Prunus domestica* L. transformed with the *Plum pox virus* strain D coat protein gene (PPV-CP) (Hily et al., 2004; Malinowski et al., 2006; Ravelonandro, 1997; Ravelonandro et al., 2000). C5 viral resistance is based on RNA silencing (Scorza et al., 2001), i.e. a sequence-specific RNA degradation mechanism widely observed in animals, fungi and plants.

Material and methods

Nine C5 transgenic plum trees grown in an experimental orchard were bud-grafted with plum cv. 'St Julien' infected with PPV-Rec strain (Polak et al., 2008). The leaf samples from the trees were collected during years 2006, 2007 and 2008. Always, three samples were collected per one tree - leaves of the 'St Julien' infectious bud (the non-transgenic part), leaves of bottom part of the C5 (leaves close to the infectious bud, transgenic part) and leaves of top part of the C5 (far from the infectious bud, transgenic part). The material was grinded in liquid nitrogen and several aliquots of 0.1g were stored at -80 °C. Thereafter, total RNA extraction was performed by RNeasy Mini Plant extraction kit (Qiagen, USA) with modification as in Mekuria et al. (2003). Concentration of the RNA samples was determined and diluted to 50 ng/ μl . All the samples were treated with DNase I (DNAfree, Ambion) and stored at -80 °C until further manipulation.

18S ribosomal RNA was used as the endogenous control for the relative quantification.

The primer pair for 18S ribosomal RNA gene Pru18SF1 5'-CGTCACACGCCGTTGCCCC-3' and Pru18SR1 5'-GAGCCGAGCATTTTTTCGAGCCC-3' amplifying a PCR fragment of 199 bp (NCBI Acc. Number [EF211087](#)) and primer pair specific for PPV-Rec targeting (Cter) NIB-(Nter) CP 8532-8669 (NCBI Acc. Number [AY028309](#)) RecJF: 5'-AATGATATTGATGATAGCCTTGAC-3' and RecJR 5'-AGCTGGTTGAGTTGTGCCAC-3' amplifying a 138bp product. Specificity of the PPV-Rec primers was checked by Real-time RT-PCR on PPV-D, PPV-M and PPV-Rec isolates (data not shown).

For the purpose of absolute quantification, a specific PPV-Rec fragment described above was inserted into the vector pGem-T (Promega Inc.) and cloned into *E. coli* JM-109. The plasmid was linearized at the *Rsa* I site and used as target in an *in vitro* transcription reaction performed with Megascript T7 kit (Ambion Inc., TX) followed by DNase I treatment (DNAfree, Ambion). The amount of RNA was quantified by UV densitometry. 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). The following mathematical formula was applied: pmol of ssRNA = $\mu\text{g (of ssRNA)} \times (106 \text{ pg}/1\mu\text{g}) \times (1 \text{ pmol}/340 \text{ pg}) \times (1/\text{Nb})$. Avogadro constant was used to estimate the number of transcripts (6.023×10^{23} molecules/mol) (Olmos et al., 2005). Subsequently, ten-fold serial dilutions of the transcripts were prepared and used. Real-time RT-PCR was performed with SYBR Green I, using a 7300 Real-time PCR System (Applied Biosystems, CA, USA) and Power SYBR Green RNA-to-CT™ 1-Step Kit (Applied Biosystem, USA) according to the recommendation of the manufacturer.

The relative ratios were calculated by a mathematical model, which includes an efficiency correction for Real-time PCR efficiency of the individual transcripts (Pfaffl, 2001). Amplification efficiency was established for each of the targets from serial dilutions of C5 plum leaves and ranged between 0.80 and 1.0. The relative viral gene's expression was then transformed into absolute values by simple proportion. All the results were then analyzed by paired t-test as well as by two way ANOVA test. Thereafter Bonferroni post-test was applied on the data to compare the value of each column (factor "position of the collected leaves") and each row factor "year". $P > 0.05$ were considered as non significant (ns), $P < 0.01$ as significant and $P < 0.001$ as extremely significant.

Results

The expression of the 18S ribosomal gene was quite stable among all the tested samples, with the C_t ranging from 15 to 18 cycles. In the previous in-house study, no significant differences in gene expression between virus-free and virus-infected plants had been recorded (data not shown). The C_t values for the PPV-Rec, on the other hand, varied greatly, ranging from 13 to 36 cycles. After calculating the relative viral gene abundance in the samples, the difference between the two most differing samples was 10 million fold. There were significant differences among the three parts of the tree. The virus was the most abundant in the non-transgenic parts of the trees, followed by the bottom parts of the transgenic trees and the scarcest in the top parts of the C5s. If the mean relative quantity of the virus in the top parts of the trees is assumed to be 1, then the mean relative quantity in the bottom parts of the trees is 1.3 and in the non-transgenic 'St. Julien' infectious bud it is 44. Furthermore, statistical analysis by two way ANOVA confirmed that the non-transgenic 'St. Julien' part of the C5 trees and the actual transgenic C5 parts varied in their PPV-Rec quantity levels. The differences between the virus level in 'St. Julien' infectious bud and the transgenic tree were found to be extremely significant in all years, independently on whether the samples were collected close to or far from the bud. On the other hand, there were no significant differences found between the virus titre in the bottom and top parts of the transgenic trees (see Table 1).

Tab. 1 Results of the Bonferroni post-test performed after two-way ANOVA statistical analysis.

		Bonferroni Post-test	
		Absolute quantification	Relative quantification
St Julien	2006	ns	***
vs	2007	**	***
C5 (Top)	2008	***	***
St Julien	2006	ns	***
vs	2007	**	***
C5 (Bottom)	2008	***	***
C5 (Top)	2006	ns	ns
vs	2007	ns	ns
C5 (Bottom)	2008	ns	ns

ns: Not significant; ** ($P < 0.01$); *** ($P < 0.001$)

For the absolute quantification, standard dilutions were obtained within a range from 1.78×10^6 to 1.78×10^1 copies of PPV-Rec partial RNA. Then correlation between the logarithm of copy number and number of cycle was found with $R^2 = 0.99$ and PCR efficiency = 104 %, and was used to evaluate the copies number of unknown samples. The determined number of PPV-Rec RNA copies varied from only 1 copy up to almost million copies in the sample. The mean determined number of virus copies in the top part of the C5 was 8500, in the bottom part of the C5 it was 23 000, and in the non-transgenic part it was more than ten times more, 240 000 PPV-Rec RNA copies (see Figure 1). Statistical

analysis confirmed that the differences between the virus level in 'St. Julien' infectious bud and the transgenic tree were found to be extremely significant in 2008, significant in 2007 and not significant in 2006, independently on whether the samples were collected close to or far from the bud. On the other hand, there were no significant differences found between the virus titre in the bottom and top parts of the transgenic trees and no significant differences were recorded for all the parts in 2006 (see Table 1).

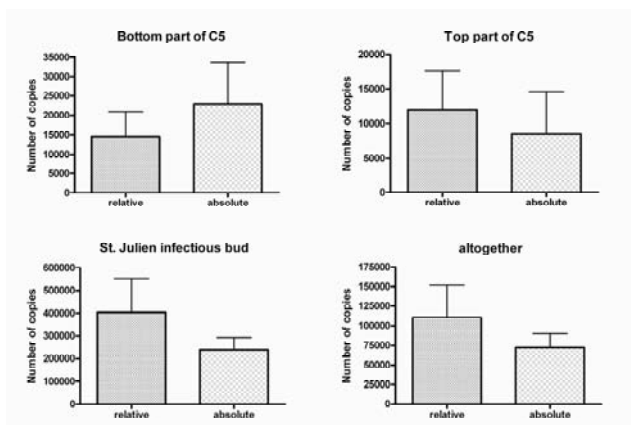


Fig. 1 Comparison of mean PPV-Rec viral RNA copies as determined by relative and absolute Real-time RT-PCR in different parts of the C5 transgenic trees sampled from 2006 to 2008.

Both methods were compared by a paired T-test, in which the numbers of viral RNA copies determined by absolute and by relative quantification were compared. In all analyses carried out (infectious buds, bottom part of C5, top part of C5, all samples together), no statistically significant differences were recorded, and in all the cases, the pairing was significantly effective. The mean values are shown in Figure 1.

Discussion

To avoid bias, relative quantification by Real-time PCR is referred to one or several internal control genes, which should not fluctuate during treatments. Ideally, the conditions of the experiment should not influence the expression of this internal control gene. However, many studies showed that internal standards could vary with the experimental conditions (Faccioli et al., 2007; Nicot et al., 2005; Sturzenbaum and Kille, 2001; Thellin et al., 1999). On the other hand, with absolute quantification, there is usually no normalizing factor and we only assume that our samples were prepared uniformly and the results are thus accurate. The drawbacks of both methods are therefore lying in normalisation. The differences between the results of the absolute and relative quantification carried out in this study were not tremendous; in almost all cases even the statistics confirmed the uniformity. However, sometimes, the statistical results reached were not in agreement, as in the case of 2006 samples. Further studies are needed in order to confirm accuracy of each method. Nevertheless, careful choice of endogenous control including stability of gene expression testing, as well as uniform and precise sample preparation for quantification by both absolute and relative quantification are highly recommended.

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