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

Jarošová, J., Gadiou, S., Kumar, J.K.
Department of Virology, Crop Research Institute, Drnovská 507, 161 06 Prague, The Czech Republic

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 contemporarily, 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 (ΔΔCT-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 leave 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'-CGTCACACGCCGTTGCCCCC-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'-AGCTGGTTGAGTTGTTGCCAC-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= µg (of ssRNA)×(106 pg/1µg)×(1 pmol/340 pg)×(1/Nb). Avogadro constant was used to estimate the number of transcripts (6.023×1023 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 of 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 of 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).

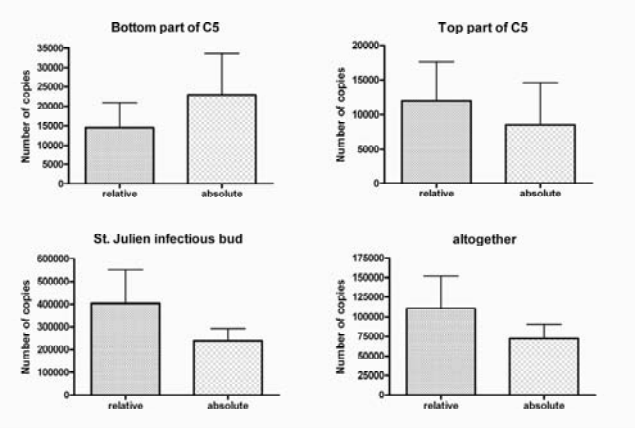

Fig. 1 Comparison of mean PPV-Rec viral RNA copies as determined by relative and absolute Real-time RT-PCR in different parts of the C5 transgenic trees sampled from 2006 to 2008.

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.

➢ This research was sponsored by Project No. MZE 0002700604.

Literature

Candresse, T., F. Rafia, J. Dunez, M. Cambra, M. Asensio, M. Navratil, J.A. Garcia, D. Boscia, G. Pasquini, and M. Barba, 1998: Characterization of plum pox virus coat protein epitopes using fusion proteins expressed in E-coli. 17Th International Symposium on Virus and Virus-Like Diseases of Temperate Fruit Crops: Fruit Tree Diseases, Vols 1 and 2, 461-467, 787.

Faccioli, P., G.P. Ciceri, P. Provero, A.M. Stanca, C. Morcia, and V. Terzi, 2007: A combined strategy of "in silico" transcriptome analysis and web search engine optimization allows an agile identification of reference genes suitable for normalization in gene expression studies. Plant Molecular Biology **63**, 679-688.

Gachon, C., A. Mingam, and B. Charrier, 2004: Real-time PCR: what relevance to plant studies? Journal of Experimental Botany **55**, 1445-1454.

Glasa, M., L. Palkovics, P. Kominek, G. Kabonne, S. Pittnerova, O. Kudela, T. Candresse, and Z. Subr, 2004: Geographically and temporally distant natural recombinant isolates of Plum pox virus (PPV) are genetically very similar and form a unique PPV subgroup. Journal of General Virology **85**, 2671-2681.

Hily, J.M., R. Scorza, T. Malinowski, B. Zawadzka, and M. Ravelonandro, 2004: Stability of gene silencing-based resistance to Plum pox virus in transgenic plum (Prunus domestica L.) under field conditions. Transgenic Research **13**, 427-436.

James, D., and A. Varga, 2005: Nucleotide sequence analysis of Plum pox virus isolate W3174: Evidence of a new strain. Virus Research **110**, 143-150.

Kerlan, C., and J. Dunez, 1979: Biological and Serological Differentiation of Strains of Plum Pox Virus. Annales De Phytopathologie **11**, 241-250.

Klein, D., 2002: Quantification using real-time PCR technology: applications and limitations. Trends in Molecular Medicine **8**, 257-260.

Mackay, I.M., 2004: Real-time PCR in the microbiology laboratory. Clinical Microbiology and Infection **10**, 190-212.

Malinowski, T., M. Cambra, N. Capote, B. Zawadzka, M.T. Gorris, R. Scorza, and M. Ravelonandro, 2006: Field trials of plum clones transformed with the Plum pox virus coat protein (PPV-CP) gene. Plant Disease **90**, 1012-1018.

Mekuria, G., S.A. Ramesh, E. Alberts, T. Bertozzi, M. Wirthensohn, G. Collins, and M. Sedgley, 2003: Comparison of ELISA and RT-PCR for the detection of Prunus necrotic ring spot virus and prune dwarf virus in almond (Prunus dulcis). Journal of Virological Methods **114**, 65-69.

Németh, M., 1986: Virus, Mycoplasma and Rickettsia Diseases of Fruit Trees Akademia Kiado.

Nemchinov, L., and A. Hadidi, 1996: Characterization of the sour cherry strain of plum pox virus. Phytopathology **86**, 575-580.

Nicot, N., J.F. Hausman, L. Hoffmann, and D. Evers, 2005: Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot **56**, 2907-14.

Olmos, A., E. Bertolini, M. Gil, and M. Cambra, 2005: Real-time assay for quantitative detection of non-persistently transmitted Plum pox virus RNA targets in single aphids. J Virol Methods **128**, 151-5.

Pfaffl, M.W., 2001: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29**, e45.

Polak, J., J. Pivalova, J.K. Kundu, M. Jokes, R. Scorza, and M. Ravelonandro, 2008: Behaviour of transgenic Plum pox virus-resistant Prunus domestica L. clone C5 grown in the open field under a high and permanent infection pressure of the PPV-Rec strain. Journal of Plant Pathology **90**, 33-36.

Ravelonandro, M., 1997: Plum trees resistant to the plum pox virus. Biofutur, 53-54.

Ravelonandro, M., R. Scorza, A. Callahan, L. Levy, C. Jacquet, M. Monsion, and V. Damsteegt, 2000: The use of transgenic fruit trees as a resistance strategy for virus epidemics: the plum pox (sharka) model. Virus Res **71**, 63-9.

Scorza, R., A. Callahan, L. Levy, V. Damsteegt, K. Webb, and M. Ravelonandro, 2001: Post-transcriptional gene silencing in plum pox virus resistant transgenic European plum containing the plum pox potyvirus coat protein gene. Transgenic Res **10**, 201-9.

Sturzenbaum, S.R., and P. Kille, 2001: Control genes in quantitative molecular biological techniques: the variability of invariance. Comp Biochem Physiol B Biochem Mol Biol **130**, 281-

Thellin, O., W. Zorzi, B. Lakaye, B. De Borman, B. Coumans, G. Hennen, T. Grisar, A. Igout, and E. Heinen, 1999: Housekeeping genes as internal standards: use and limits. Journal of Biotechnology **75**, 291-295.

Varga, A., and D. James, 2005: Detection and differentiation of Plum pox virus using real-time multiplex PCR with SYBR Green and melting curve analysis: a rapid method for strain typing. J Virol Methods **123**, 213-20.

Wetzel, T., T. Candresse, M. Ravelonandro, R.P. Delbos, H. Mazyad, A.E. Aboul-Ata, and J. Dunez, 1991: Nucleotide sequence of the 3'-terminal region of the RNA of the El Amar strain of plum pox potyvirus. J Gen Virol **72 (Pt 7)**, 1741-6.