Expression of the coat protein genes of PNRSV and PDV in the synergistic disease peach stunt

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Abstract

Simultaneous infections of peach (*Prunus persica* Batsch L.) with the two ilarviruses, Prunus necrotic ringspot virus (PNRSV) and Prune dwarf virus (PDV), produce a synergistic disease referred to as "peach stunt". Previous work showed significant differences in the expression of the coat protein (CP) genes. In the presence of PNRSV, an up to 17-fold reduction in the amount of (+) strand RNA 3 of PDV, as compared to similar trees infected with PDV alone, was observed. However, the presence of PDV had no effect on the concentration of (+) strand RNA 3 of PNRSV (Scott et al., 2001). This work re-examines and extends these observations using multiplex real-time PCR. Probes to both the plus and minus strands of the RNA 3 of each virus were designed and synthesized. The comparative C_T method ($\Delta\Delta C_T$) was used for relative quantitation of gene expression. A reduction in the amount of (+) strand RNA 3 of PDV observed in the earlier work was not seen using real-time PCR. However, in a time course experiment with samples collected at 14-day intervals for 6 weeks, there was a substantial increase in the concentration of the (+) strand of RNA 3 of PDV after 14 days irrespective of whether the virus was present as a sole infecting agent or as a co-infection with PNRSV. At this same point in time there was a decrease in concentration of (+) of the RNA 3 of PNRSV. By the next sample date the concentrations of the (+) strand of RNA 3 of both viruses had returned to "normal". The results are discussed in relation to the most extensively studied plant viral synergism synergism (PVY and PVX) and to known changes in concentration of PNRSV and PDV based on earlier observations made using ELISA.

Keywords: Peach, Prunus necrotic ringspot virus, Prune dwarf virus, peach stunt disease, molecular interaction, Multiplex real-time PCR.

Introduction

The infection by plants with two or more unrelated viruses/virus-like agents is a common phenomenon in nature particularly in long lived, perennial species such as fruit trees (Nemeth, 1986). Many such infections result in a synergistic interaction that is visibly manifest by a remarkable change in symptom expression, yield loss, and/or host longevity. In addition, in well studied synergies a change in virus accumulation of one of the participant viruses has been documented. Many synergistic interactions involve a member of the potyvirus group and viruses belonging to other families. In most of these interactions, the accumulation level of the potyvirus remains unaltered, while the level of accumulation of the nonpotyvirus increases considerably (Pruss et al., 1997; Vance, 1991).

The most extensively studied synergistic interaction involves Potato virus Y (PVY, the type member of the genus Potyvirus, family *Potyviridae*), and Potato virus X (PVX, the type member of the genus *Potexvirus*, family *Flexiviridae*) in tobacco (Vance, 1991). In plants co-infected with both PVY and PVX, accumulation of PVX in systemically infected leaves increases up to 10-fold compared to equivalent leaves of singly infected plants. The level of PVY is the same irrespective of whether the plant is infected with PVX. In addition, the accumulation of PVX (–) strand RNA was much higher in doubly infected cells than was PVX (+) strand RNA, indicating that PVY induced a change in the regulation of PVX replication (Pruss et al., 1997; Vance, 1991). Moreover, it has since been demonstrated that replication of the potyvirus is not required for potyviral/PVX synergism, as the response is mediated by 5' potyviral sequences comprising P1 and helper component proteinase proteins (P1/HCPro) (Pruss et al., 1997; Vance et al., 1995). P1/HC-Pro also functions as a pathogenicity enhancer and activator of replication of other unrelated viruses such as Cucumber mosaic virus (CMV, the type member of the genus *Cucumovirus*, family *Bromoviridae*). In co-infections, PDV and PNRSV (both members of the genus *Ilarvirus*, family *Bromoviridae*) may interact in either a synergistic or an antagonistic fashion (Cropley, 1968). In peach (*P. persica* L. Batch), infection with both viruses results in a disease known in Australia as peach rosette and decline (Smith and Challen, 1976) and in California as peach stunt disease (Asai and Uyemoto, 1991). Symptoms of the two diseases include stunting, yellowing, and a 2 to 6-fold reduction in fruit yield. In both diseases the effects on growth and yield are typically greater than infections with either virus alone and are greater than the additive effects of infections by individual viruses. In the presence of PNRSV, an up to 17-fold reduction in the amount of (+) strand RNA 3 of PDV, as compared to similar trees infected with PDV alone, has been observed. However, the presence of PDV had no effect on the concentration of (+) strand RNA 3 of PNRSV (Scott et

al., 2001). These results would indicate a significant reduction in the accumulation of PDV in the plant when PNRSV was present but that accumulation of PNRSV is unaffected by the presence of PDV. In this way the effects on the accumulation of PNRSV mimic the effects observed on the accumulation of PVY in the PVY/PVX synergism in tobacco but the effects observed on the accumulation of PDV are in complete contrast to those observed for PVX. However, unlike PVY and PVX, PDV and PNRSV are members of the same viral genus (*Ilarvirus*), both possess a tripartite genome, and neither code for a molecule similar to P1/HCPro. Nor do these two particular ilarviruses possess the ORF found in some other ilarviruses and the cucumoviruses that codes for the 2b protein shown to be involved in the suppression of RNA interference (Lucy et al., 2000). A role that is associated with the HCPro protein of potyviruses. Thus, although the biology of the synergism between PDV and PNRSV is well documented, information on the regulation of the synergism at the molecular level is absent. In this work we attempt to confirm earlier findings concerning the accumulation of the viruses and assess if either virus affects the replication of the other through examining the production of minus strand RNA 3. Unique probes to the CP genes of PDV and PNRSV and to the plus and minus strands of the RNA 3 of each virus were used in one-step, multiplex, real-time PCR to simultaneously detect up to three target RNAs in a single sample.

Materials and methods

Preparation of RNA: Total RNA was extracted from leaves of small trees of the peach cultivar 'Juneprince' growing on the rootstocks, 'Lovell', 'Nemaguard' and 3-17-7 (Guardian®) using a procedure modified from Hughes and Galau (1988). Samples were collected from a single bud at 2-week intervals for a period of 6 weeks beginning as leaves emerged from the dormant buds in the spring. The RNA was extracted from 100 mg samples of leaf tissue and the yield of nucleic acid per sample (µg/µl) determined by UV spectophotometry. Integrity of the sample was confirmed by gel electrophoresis in 2 % agarose gels buffered with TAE and with the sample having been denatured using formamide. Aliquots (5 µl) of each sample containing 190 ng of total RNA were prepared and stored at -80 °C until added to a real-time PCR reaction so as not to subject the RNA sample to cycles of freezing and thawing.

Real-time PCR: Real-time PCR was completed using a QIAGEN QuantiTect Multiplex RT-PCR NR Kit according to the manufacturer's instructions. Probes to plus strand RNA 3 and minus strand RNA 3 of the movement protein gene, and to the coat protein gene (RNA 3, RNA 4) were used to detect the expression of the RNA 3. Probes for PDV were labeled with FAM at the 5' terminus and quenched at the 3' terminus with BHQ 1. Probes for PNRSV were labeled with Quasar 670® at the 5' terminus and quenched at the 3' terminus with BHQ 2 (Biosearch Technologies, Novato, CA). Sequences of primers and probes used in this work are shown in Table 1.

Tab. 1 Sequence of the primer pairs and probes used to detect plus and minus strand RNA 3 and the coat proteins of PDV and PNRSV.

PDV Movement protein	Forward primer	5' AAGCGGCTATCTTCGTTGGAA 3'
Plus strand	Reverse primer	5' GCGTCTACACTTACGGCTGAT 3'
	Probe	5' ATGGCCAAGAGCAGTTCACGCC 3'
PDV Movement protein	Forward primer	5' CCTTTCCACTACTCCCAATTACCA 3'
Minus strand	Reverse primer	5' CCCTTGCTGCTGTAGATGATGTG 3'
	Probe	5' CGCGATTTGGCGAATGTTTGGAGTAT 3'
PNRSV Movement protein	Forward primer	5' CCGACAGGCCGATAAAGTAAAGAAG 3'
Plus strand	Reverse primer	5' CGAGGTCGTTGCTTGAATGATC 3'
	Probe	5' TTACAGATGTGTAGGCCGAGTATTCC 3'
PNRSV Movement protein	Forward primer	5' CCTTCTGTACCTGCCAATATCCTA 3'
Minus strand	Reverse primer	5' TTCCCGATTGCCGAGACAA 3'
	Probe	5' TCGGACCATAGACATCAACACCTTC 3'
PDV Coat protein	Forward primer	5' TGATACCAAGGTRTACGGAATYG 3'
	Reverse primer	5' TGAACTTCCTACGTTGTAGGGGATT 3'
	Probe	5' TCTAYGGACTCATTAAAGGT 3'
PNRSV Coat protein	Forward primer	5' CCKCAGTTGATGGGTCAGAATTT 3'
	Reverse primer	5' CCTTCAAGAACCCCTTCCTAGAC 3'
	Probe	5'CCGAATGAACTCTATGAGTTCGAATGGTTGG 3'

Experimental design and analysis: Experiments were set up in 96-well plates using 5 replicates of 16 treatments arranged in a balanced lattice design (Burrows et al., 1984). The reactions were performed in a Stratagene Mx 3000p real-time thermocycler using ROX as the passive reference dye. CT values for different treatments were analyzed using the GLM procedure of SAS (SAS Institute Inc, Cary, NC, USA) and significant differences among means of treatments identified.

Results

Multiplex real-time PCR was able to detect the segments of the RNA 3 of both PNRSV and PDV to which the probes were designed, specifically, unambiguously, and with equal efficiency in the same sample, thus allowing the comparison of the relative expression of the genomic molecules using the comparative C_T method ($\Delta\Delta C_T$) (Dorak, 2006). Typical results are shown in Table 2. In no instances were the C_T values obtained for a virus in a plant infected with both viruses (PDV + PNRSV) significantly different from the C_T values obtained for the virus (PDV or PNRSV) when infecting the plant alone. The presence of PDV was not detected in total RNA samples collected at the start of this experiment even though the PCR reaction was detecting PDV in positive controls (C_T counts of 11). However, samples collected two weeks into the time course experiment showed levels of PDV (average C_T counts of 16.9) for the CP similar to those detected throughout the remainder of the time-course experiment. The levels of PNRSV detected in these experiments (C_T values 15 – 20.1) were consistent throughout the entire 6 weeks of the experiments. No significant differences between the C_T values detected for plus or minus strands of the RNA 3 of either virus were detected. In non-inoculated/non-infected trees no detectable C_T values were recorded before the maximum number of cycles (45) in an experiment were completed. In preliminary experiments, no detectable C_T values were recorded in non-inoculated/non-infected trees even when the maximum number of cycles used for amplification was increased to 55.

Tab. 2 C_T values recorded for one-step, real-time PCR reactions completed using RNA extracted from samples collected from the scion cultivar 'Juneprince' 2 weeks into the time course experiment. Mean C_T values of 5 replications for each virus rootstock combination are shown. The columns labeled PNRSV/PDV show the C_T values detected for PNRSV and PDV, respectively, in trees in which both viruses were present. Each reaction contained 190 ng of total RNA. ROX dye was used as the passive reference dye. Positive controls using 190 ng of purified virus gave C_T values of 11 or 12.

Mean CT values for minus strand RNA 3

	Virus treatment			
	PNRSV	**PDV**	**PNRSV/PDV**	**Noninoc**
Root stock				
Lovell	20.0	18.8	20.3/17.8	>45
Nemaguard	19.9	18.5	20.6/17.4	>45
Guardian	20.1	17.9	20.2/18.8	>45

Mean CT values for coat protein gene (RNA 3 and RNA 4)

	Virus treatment			
	PNRSV	**PDV**	**PNRSV/PDV**	**Noninoc**
Root stock				
Lovell	15.0	14.9	15.6/14.8	>45
Nemaguard	15.0	18.1	16.2/17.8	>45
Guardian	16.2	17.9	17.2/18.8	>45

	Virus treatment			
	PNRSV	**PDV**	**PNRSV/PDV**	**Noninoc**
Root stock				
Lovell	17.3	19.5	17.3/20.8	>45
Nemaguard	16.2	19.3	17.0/19.0	>45
Guardian	16.1	19.5	16.3/19.8	>45
Guardian	16.2	17.9	17.2/18.8	>45

Discussion

In an initial examination of this synergism (Scott et al., 2001), the presence of PNRSV led to a 17 fold reduction in the accumulation of PDV as detected using northern blots and cRNA probes to the CP gene (RNA 3 and RNA 4). The presence of PDV had no effect on the accumulation of PNRSV. In this respect the absence of changes in the accumulation of PNRSV is similar to that observed for PVY, whereas the reduction in the accumulation of PDV is in complete contrast to the observed effects on PVX. Furthermore it was clear that PVY affected the replication of PVX (Vance, 1991). In this work we were unable to duplicate the reduction in accumulation of PDV achieved earlier. The concentrations of PNRSV were unaffected by the presence of PDV and there were no effects on the replication of either virus as judged by the relative levels of minus strand RNA 3 that were detected for each virus. Although not shown in the data presented, there appears to have been a delay in the replication /accumulation of PDV in buds at the beginning of this experiment.

The major difference between this series of experiments and those completed previously are the genotypes of the material used. In the previous experiment (Scott et al., 2001), the scions 'Garnet Beauty', 'Elberta', and 'GF 305' growing on the rootstocks Lovell and Nemaguard were used. The reduction in accumulation of PDV occurred in 'Elberta' and 'GF305'. Both of these cultivars are recommended as susceptible woody indicators for a number of stone fruit viruses and virus-like diseases providing good symptom expression in the greenhouse in particular. It is possible that the genotypes of these two cultivars are responsible for the reduction in accumulation of PDV. Now that reliable, one-step multiplex real time PCR has been developed for these two viruses, it will be possible to re-examine the original work using 'Elberta' and 'GF305'. The two viruses can be detected simultaneously in a single sample and, within the limitations of current technology, paired comparisons of the accumulation of the plus and minus strands of all three genomic molecules can be completed in the hopes of identifying the molecules that interact in this non potyvirus synergism.

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