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Stability of gene silencing-based resistance to *Plum pox virus* in transgenic plum (*Prunus domestica* L.) under field conditions

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Abstract

Plum pox *virus* (PPV) is one of the most devastating diseases of *Prunus* species. Since few sources of resistance to PPV have been identified, transgene-based resistance offers a complementary approach to developing PPV-resistant stone fruit cultivars. C5, a transgenic clone of *Prunus domestica* L., containing the PPV coat protein (CP) gene, has been described as highly resistant to PPV in greenhouse tests, displaying characteristics typical of post-transcriptional gene silencing (PTGS). We show in this report that C5 trees exposed to natural aphid vectors in the field remained uninfected after 4 years while susceptible transgenic and untransformed trees developed severe symptoms within the first year. C5 trees inoculated by chip budding showed only very mild symptoms and PPV could be detected in these trees by IC-RT-PCR. The PPV-CP transgene in C5 was specifically hyper-methylated with no detectable expression. These results indicate both stability and efficiency of PTGS-based PPV resistance in plum under field conditions.

Introduction

Most cultivated *Prunus* species are highly susceptible to 'sharka' or plum pox disease, caused by *Plum pox virus* (PPV) (PPV: family *Potyviridae*, genus *Potyvirus*). Originally reported in Bulgaria (Atanassov, 1932), the virus has progressively spread throughout Europe, the Mediterranean basin (Al Rwahnih et al., 2001), South America (Rosales et al., 1998), and most recently North America (Levy et al., 2000; Thompson et al., 2001). PPV is spread naturally by several aphid species in a non-persistent manner (Kunze and Krczal, 1971). It causes one of the most devastating diseases of stone fruits (Dunez and Sutic, 1988). Currently, PPV control relies mainly on prevention by the use of certified plant material for new orchards, quarantine measures, and eradication.

Prunus (peach, plum, apricot and cherry) cultivars have been described as 'susceptible,' 'tolerant,' 'resistant,' or 'immune' to PPV, but contradictory evaluations have made the interpretation of these ratings problematic (Kegler et al., 1998). Few highly resistant cultivars have been developed. The difficulty in producing highly resistant cultivars through conventional plant breeding suggests the utility of pathogen-derived resistance (Sanford and Johnston, 1985) for providing virus resistance (Powell et al., 1986; Beachy et al., 1990; Wilson, 1993; Baulcombe, 1996).

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Ravelonandro et al. (1993) demonstrated pathogen-derived PPV resistance in the herbaceous model *Nicotiana benthamiana* using the coat protein (CP) gene of PPV. Based on this work, plum (*Prunus domestica* L.) was transformed with the PPV-CP gene (Scorza et al., 1994). Transgenic plum clones were inoculated with PPV and one line, C5, was found to be highly resistant to infection. Transgenic clone C5 remained symptomless and ELISA negative for PPV (Ravelonandro et al., 1997, 1998a), but the presence of the virus in graft-inoculated C5 plants could be detected by IC (immunocapture)-RT-PCR (Scorza et al., 2001). Scorza et al. (1994) demonstrated that C5 contained multiple transgene copies. PPV-CP transcript levels were high in the nucleus but low in the cytoplasm, and no CP was detected. The PPV-CP transgene was shown to be specifically methylated (Scorza et al., 2001) demonstrating post-transcriptional gene silencing (PTGS)-based resistance as described in herbaceous systems (Hobbs et al., 1993; Lindbo et al., 1993; Ingelbrecht et al., 1994; English et al., 1996; Stam et al., 1997).

C5 was the first demonstration of PTGS-based virus resistance in a temperate woody perennial. The potential for using PTGS as a technology for producing virus resistant woody perennial species depends upon the stability of resistance in the field over the productive life of an orchard or a clone. To validate the stability of PTGS-based PPV resistance in C5, fields tests were undertaken. Preliminary field trial results were described (Malinowski et al., 1998). In this report we show the stability of PTGS in C5, as measured by low level PPV-CP RNA accumulation, elevated specific methylation, and resistance to PPV after 4 years in the field.

Materials and methods

Transgenic plum lines, plant culture conditions, and inoculation with PPV

Five transgenic plum clones (C2, C3, C4, C5, C6) previously described (Scorza et al., 1994; Ravelonandro et al., 1997, 1998a) and untransformed control plum B70146, all grafted onto GF8-1 rootstocks, were planted in April 1996 in a newly established field trial, approved by the Polish Ministry of Environment, in the experimental field of the Research Institute of Pomology and Floriculture near Skierniewice, Poland (Malinowski et al., 1998). In August 1996, 2 of 10 trees of each clone were inoculated by chip bud inoculation (CBI) as described by Malinowski et al. (1998) with PPV-S, a D type isolate of PPV. The other eight trees of each clone were left for inoculation through aphid vectored inoculation (AVI). Trees of 'Sweet Common Prune' (syn. 'Hauszwetsche') were planted in rows alternating with rows of test trees. These 'Sweet Common Prune' trees were CBI with PPV-D to provide ample sources of inoculum for aphid acquisition of PPV. Also, naturally PPV-infected plum trees grown in close vicinity of the field test (within 50 m) provided additional sources of inoculum.

Evaluation of PPV infection

PPV infection of test trees was evaluated by visual observation of symptoms, ELISA, RT-PCR or IC-RT-PCR. Briefly, symptoms were evaluated each month throughout the growing season. Standard double-antibody sandwich ELISA for PPV was performed two to three times per growing season. RT-PCR amplification of the fragment of the PPV nuclear inclusion body (NIb) cistron using the primers PPV-A, PPV-B (Korschineck et al., 1991) was performed when necessary to confirm ELISA results as described by Malinowski et al. (1998). IC-RT-PCR with the same primer pair was also used to confirm the absence of infection in some trees (Malinowski et al., 1998).

Sampling, DNA and RNA isolation

Three classes of experimental trees were selected for our study: transgenic PPV-resistant (C5), transgenic PPV-susceptible (C2, C3, C4 and C6), and non-transgenic susceptible (B70146). Within each clone, two sub-treatments were selected, CBI and AVI. Leaf samples were collected randomly throughout the canopy from branches of similar age for evaluation of PPV infection. Further molecular analyses were conducted on one sample from a randomly selected tree of each treatment (AVI, CBI) for C3, C5, and B70146.

Leaves for nucleic acid extraction were collected from trees 4 years post-planting, in June 2000 (June 6, 2000 for all data, except extracts for simultaneous ELISA and IC-RT-PCR which were sampled on June 13, 2000). Since aphid inoculation occurred naturally, the timing of aphid inoculation could not be determined. Leaves were immediately frozen in liquid nitrogen and stored at −80°C. Leaves of infected control plants were obtained from the USDA-ARS quarantine facility at Ft. Detrick, MD. These were infected with the M strain of PPV as described previously (Ravelonandro et al., 1997; Scorza et al., 2001). Uninfected controls were sampled from greenhouse or field-planted trees grown at USDA-ARS, Kearneysville, WV. DNA and RNA were extracted from leaf samples, as previously described (Verwoerd et al., 1989; Kobayashi et al., 1998).

DNA methylation analysis

Methylation status was evaluated as previously described (Ingelbrecht et al., 1994; Scorza et al., 2001). Briefly, restriction digestion of 5 µg of genomic DNA was carried out overnight at 37°C using 1 unit/µg for each enzyme (methylation sensitive *Sau*3AI and methylation insensitive *Mbo*I) (Gibco BRL, Life Technologies) in the presence of 5 mM spermidine in a volume of 200 µL.

Quantitative PCR was performed using an ABI Prism 7900HT sequence detection system with 2 × SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA).

The PCR reactions were performed in triplicate for each sample. Real time amplification plots were used to determine the threshold cycle number (C_t), which is the cycle at which a significant increase in amplification (as measured by binding SYBR® Green to specific product) is first detected (TaqMan PCR protocol, Applied Biosystems). Since cytosine methylation will inhibit *Sau*3AI digestion, the higher the level of methylation, the higher the amplification value. Values were quantitatively standardized to take into account the level of digestion. A dissociation curve analysis was performed after each run to screen for non-specific products. New primers were specifically designed by Primer express 2.0 software for methylation analysis by TaqMan quantitative PCR (Table 1).

Table 1. Oligonucleotide sequences used to amplify regions of the transgene insert and native chlorophyll *a*/*b*-binding protein gene in plum

	Oligonucleotide sequences	
Designation	Technique	Sequence
gusA 1320 fwd	TaqMan	CGGAAGCAACGCGTAAACTC
gusA 1257 rev	TaqMan	TGAGCGTCGCAGAACATTACA
gusA 1367 fwd[a]	TaqMan	AGGTGCACGGGAATATTTCG
gusA 1320 rev[a]	TaqMan	ACGCGTCGGGTCGAGTT
35S-gusA fwd	TaqMan	CGCAATGATGGCATTTGTAGG
35S-gusA rev	TaqMan	GATTTCAGGGGTTGGGGTT
35S-PPVCP fwd	TaqMan	ACGTAAGGGATGACGCACAAT
35S-PPVCP rev	TaqMan	CTCGTCCTCTCTTTCGTCAGC
PPV-CP 340 Sau3AI fwd	TaqMan	CAACTCAAACGCGCTAGTCAAC
PPV-CP 340 Sau3AI rev	TaqMan	GGCACTGTAAAAGTTCCACTTGATC
PPV-CP 660 Sau3AI fwd	TaqMan	GGGAAACACAAGTGGAGTATCCA
PPV-CP 660 Sau3AI rev	TaqMan	ATACGCTTCAGCCACGTTACTG
PPV-CP 62 fwd[a]	TaqMan	GCAGGCAAGCCGATTGTAGT
PPV-CP 132 rev[a]	TaqMan + RT-PCR	TGTATGACTGGAGGTGGTTGAAGT
Cab fwd	TaqMan	CTATCTTGGCAACCCAAACCT
Cab rev	TaqMan	GTGGATCCAGTCCTTCACCAA
PPV-CP transgene fwd	TaqMan + RT-PCR	CGTTTTAAATATGGCATGCCAAA

[a] These primers span sequences that do not contain a *Sau3*AI site. Data developed from these amplifications were used for standardization.

RT-PCR

Following DNase treatment (DNase I-RNase-free, 1 unit/μg, Roche, Mannheim, Germany), RT-PCRs were conducted in a one step reaction using the GenAmp® EZrTth RNA PCR Kit (Applied Biosystems) from 100 ng of total RNA, in 25 μL of reaction according to the manufacturer's instructions. Samples were heated at 60°C for 30 min (reverse transcription phase), and after 1 min at 94°C, samples went through 40 cycles 94 (15 s) – 60°C (30 s). Following cycling, 7 min at 60°C completed the extension. Specific primers were used to detect M and D strains of PPV (previously described by Olmos et al., 1997). Also, specific primers to distinguish viral from transgene PPV-CP mRNA (see Table 1) were used as described in Ravelonandro et al. (1992). RNA quality was verified by amplification of 18S ribosomal RNA.

Results

Plum pox virus *infection*

All eight trees of clone C5 exposed to AVI remained healthy in the field after four growing seasons, based on symptomology, ELISA and IC-RT-PCR analyses. These results confirmed greenhouse tests (Ravelonandro et al., 1997, 1998a). In contrast, most trees of other clones became infected under the same conditions (Table 2). Susceptible transgenic clones and non-transformed trees in the test block, both CBI and those trees left to AVI, began exhibiting symptoms the first summer after inoculation or exposure to aphids in the field, with an increasing number of trees becoming infected yearly. By the end of the 40 year in the field, 95% of these sensitive trees were infected as determined by symptom expression (Figure 1a,b) and ELISA (Figure 1d). Although highly resistant, C5 trees were not immune to PPV. The two C5 trees that were CBI displayed mild symptoms (Figure 1c) on a few leaves on single branches starting from the second year after inoculation. Symptoms appeared on CBI C5 1 year later than for the other CBI trees. By year four, CBI C5 trees produced no symptomatic leaves, but during the growing season a few samples were ELISA positive. ELISA readings were nevertheless consistently lower for CBI C5 than for susceptible clones whether the susceptible clones were CBI or AVI (Figure 1d). The June 2000 CBI some C5

Table 2. Infection of transgenic clones of plum by *Plum pox virus* (PPV), after 4 years in the field

Clone	Number of infected trees[a]			
	Inoculation by chip budding		Natural aphid vectored inoculation[b]	
	No. trees infected/No. trees tested	Symptom severity	No. trees infected/No. trees tested	Symptom severity
B70146	2/2	3	7/7[c]	3
C2	2/2	2	8/8	2
C3	2/2	2	8/8	2
C4	1/1[c]	2	6/8	2
C5	2/2	1	0/8	0
C6	2/2	2	8/8	2
Total	11/11		37/47	

[a] a tree was declared infected when specific leaf symptoms were present and a sample from the tree was found positive for PPV by ELISA, RT-PCR or IC-RT-PCR.

[b] the experimental orchard was interplanted with PPV infected non-transgenic trees to provide virus inoculum for aphids naturally present in the orchard.

[c] one tree died due to non-virus related causes.

0 no symptoms, tested negative for PPV by ELISA, RT-PCR and IC-RT-PCR.

1 Very mild and delayed symptoms. Few leaves with symptoms could be found on the tree. Low concentrations of virus particles could occasionally be detected by ELISA, and generally by IC-RT-PCR.

2 typical symptoms appeared within the first year post-inoculation. Tree tested ELISA positive for PPV.

3 severe symptoms visible at distance within the first year post-inoculation.

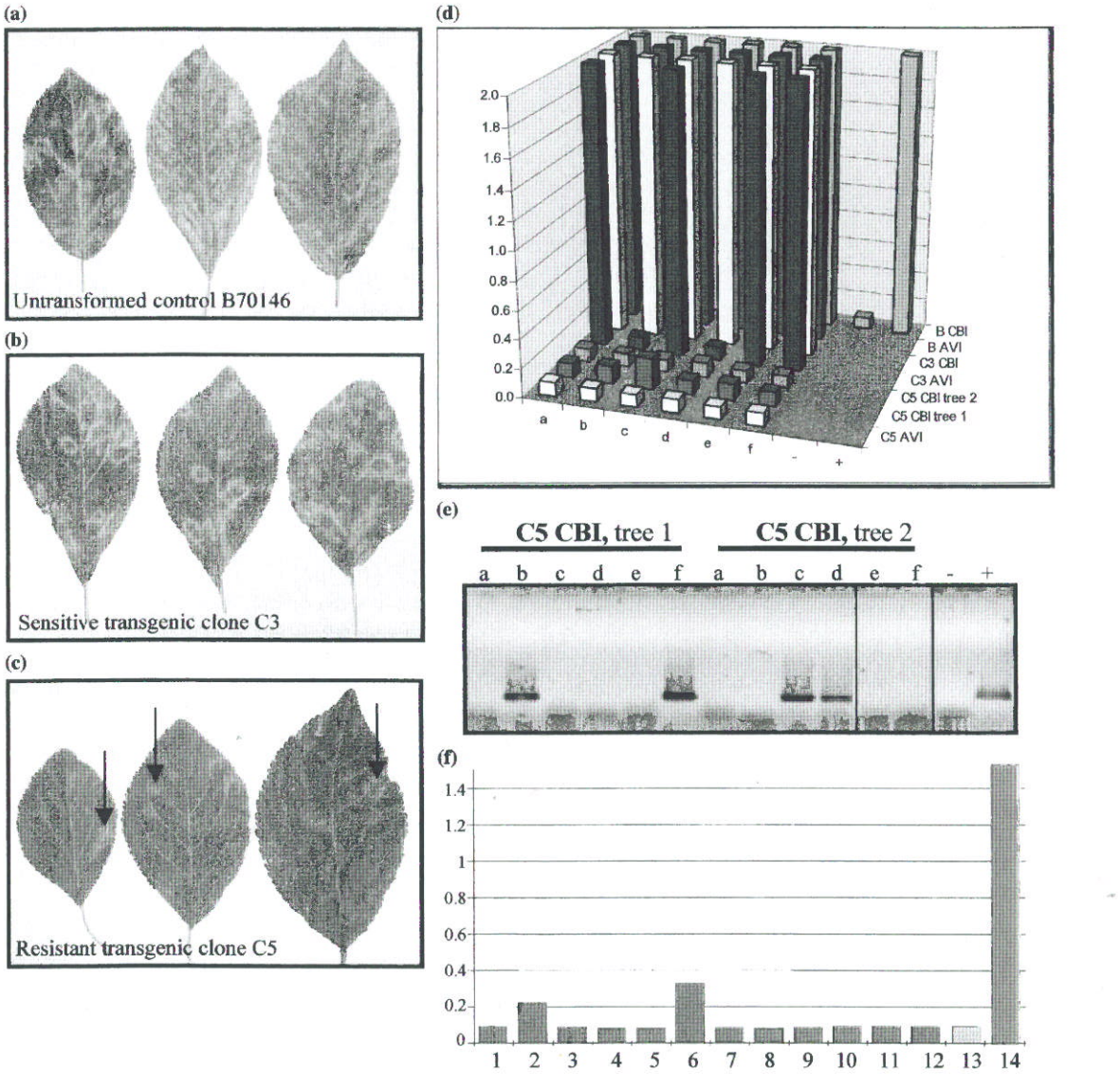

Figure 1. Detection of *Plum pox virus*. (a) Symptoms of PPV infection on CBI untransformed clone leaves, (b) CBI susceptible transgenic C3 clone and (c) very mild symptoms on CBI C5 (indicated by arrows). (d) ELISA analysis of six separate leaf samples for each tree (sampled on 6/6/2000). Simultaneous (e) IC RT-PCR and (f) ELISA analysis of both CBI C5 trees (sampled on 6/13/2000). a,b,c,d,e,f, six samples extracted from leaves from six different branches of a single tree; B, B70146, untransformed trees; CBI, chip bud inoculation; AVI, aphid vectored infection; –, Negative control; +, positive control.

tree samples tested were ELISA and RT-PCR negative for PPV, but PPV was detected by IC-RT-PCR (compare Figures 1d–f and 2b, c) generally on symptomatic leaves or in a few cases on asymptomatic leaves from branches that produced symptomatic leaves in the previous year. Analyses of symptom development, ELISA, and molecular determinations of infection separated clones in this field trial into four groups after 4 years of growth. (i) A highly resistant class consisting of C5 with no apparent AVI. CBI C5 trees displayed very mild and delayed symptoms (Figure 1c), were generally ELISA and RT-PCR negative (Figures 1d, 1f, 2b and 2c), IC-RT-PCR positive (2–6 samples for both C5 CBI trees, Figure 1e). (ii) Resistant C4 with 3 of 8 trees exposed to natural infection symptomless, ELISA negative

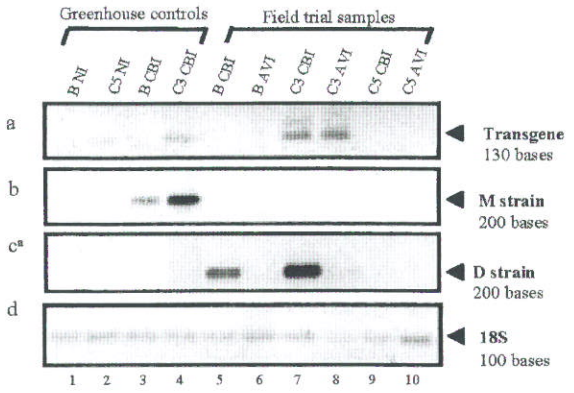

Figure 2. Detection by RT-PCR of transgene RNA and viral RNA accumulation. (a) RT-PCR analysis of transgene and (b and c) viral RNA accumulation in plum leaves. (d) RT-PCR analysis of 18S RNA. Position and expected sizes of the product are indicated. [see Ravelonandro et al. (1992)]. B, B70146, untransformed trees; NI, non-inoculated; CBI, chip bud inoculation; AVI, aphid vectored infection. [a]The primers used for amplification in transgenic clone C3 amplify both transgene and viral RNA.

and IC-RT-PCR negative for two trees (Table 2). (iii) Susceptible C2, C3, and C6 with all showing symptoms except for one C2 tree and all trees ELISA and IC-RT-PCR positive. (iv) Highly susceptible B70146 displaying severe chlorotic symptoms visible from a distance (Figure 1a), ELISA (Figure 1d) and IC-RT-PCR positive (data not shown). This grouping of clones generally followed the classification expected from greenhouse trials (Ravelonandro et al., 1997, 1998a) except for the addition of group (ii) which takes into account the partial resistance of clone C4 revealed in this study.

Accumulation of viral and transgene PPV-CP mRNA

RT-PCR confirmed in field samples the difference in transgene expression between PPV-susceptible transgenic clone C3 and the resistant transgenic C5 clone (Figure 2a). C5 had little to no detectable transgene RNA, maintaining in the field a low level accumulation of transcript RNA as had been shown in previous greenhouse studies (Scorza et al., 1994, 2001). In PPV-susceptible control plants a difference in the levels of viral RNA between AVI and CBI treatments was detected. The PPV-CP product, specific to the virus, that was detected in graft-inoculated plants of B70146 was considerably higher than in AVI plants (Figure 2c, lanes 5 and 6). This difference was also observed in C3 trees. While the PPV-CP transgene-specific primers amplified the PPV-CP transgene equally in AVI and CBI C3 samples (Figure 2a, lanes 7 and 8), the PPV-D-specific primers, which would amplify both transgene and viral RNA clearly detected a much higher level of PPV RNA in the C3 CBI sample over the AVI sample (Figure 2c, lanes 7 and 8) apparently due to viral RNA production.

Transgene methylation

PCR amplification of the entire PPV-CP sequence, following restriction enzyme digestion with methylation sensitive *Sau*3AI and isoschyzomer methylation insensitive *Mbo* I revealed that the transgene in both CBI and AVI C5 trees was methylated and that the transgene in C3 was not (data not shown). Methylation in *gusA* and *npt*II genes was not apparent in either C3 or C5 samples (data not shown).

To further analyze methylation, the same digested DNA samples used for the above PCR analyses were examined by real time quantitative PCR (TaqMan, Applied Biosystems). This method allowed the PCR to be monitored and permitted quantification. Quantitative PCR was performed by using primer-SYBR® Green combinations that spanned the *Sau*3AI restriction sites that were under evaluation. One control sequence did not contain *Sau*3AI site and was used as a reference for quantification. The amplification values were obtained from the C_t. The C_t value is inversely proportional to the amount of amplifiable starting material, and thus amplification values are derived from the inverse log of the C_t value (TaqMan PCR protocol, PE Applied Biosystems). Following the Standard Curve method (TaqMan PCR protocole, PE Applied Biosystems), the level of amplifiable DNA, and therefore methylation at the 340 *Sau*3AI site of C5, was 11–24 times greater than in DNA from C3 at the same site (Figure 3). At the 660 *Sau*3AI site, methylation was approximately 13 times greater in samples from C5 tissue than in samples from C3 tissue. No significant difference in amplifiable product between C3 and C5 was found for the *gusA* gene, and for the chlorophyll *a/b* protein gene (*cab*), which was not related to the transgene

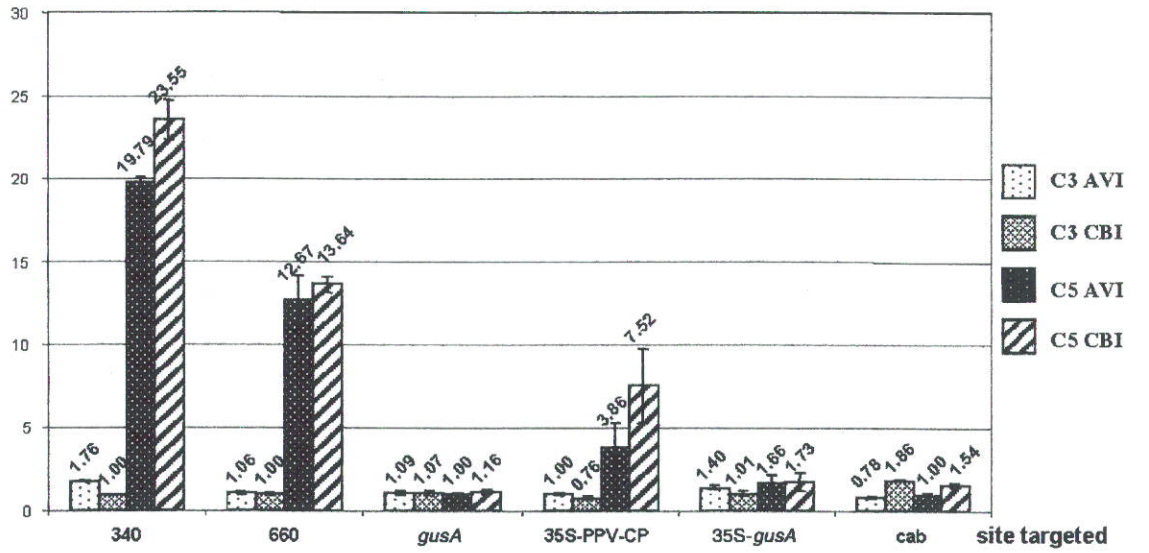

Figure 3. Relative methylation quantification by PCR (TaqMan). The *x*-axis indicates the restriction sites that were evaluated for methylation: sites 340 and 660 were in the PPV-CP sequence; *gusA* was in the *gusA* gene; 35S-PPV-CP and 35S-*gusA* sites were at the junction of the PPV-CP or *gusA* gene and its 35S promoter, respectively; cab was in the chlorophyll *a*/*b* binding protein gene which was used as a non-methylated native gene control. Values represent levels of amplification obtained from the threshold cycle number. There is a linear relationship between the amplification value and the amount of amplifiable starting material. The greater the level of DNA methylation, the higher the amplification value. Values are the average of at least three independent experiments. Error bars indicate ±SD. CBI, chip bud inoculation; AVI, aphid vectored infection.

sequence. For the CaMV promoter (35S) driving the *gusA* gene, both clones, C3 and C5, whether CBI or AVI, presented the same degree of methylation (Figure 3, lane 35S-*gusA*), while the *Sau*3AI site in the 35S promoter of the PPV-CP gene in C5 was 4–10 times more methylated than in C3 (Figure 3, lane 35S-PPV-CP).

Discussion

Although transgenic plants contribute significantly to the production of some major crop species such as soybean, maize, and cotton, especially in the US, Argentina, Canada, and China (http://www.isaaa.org/kc/bin/cbtupdate/index2.htm), the utilization of genetic engineering for other crops, especially fruit trees, lags far behind. For example, of the 8906 field releases of transgenic plants in the United States from 1987 to 2002, only 129 (< 1.5%) have been woody perennial fruit trees, nuts, or vines (http://www/osb.vt.edu/2002/menu/regulatory_information.cfm). We know of no commercial plantings of transgenic temperate woody perennial fruit trees that exist at this time. In order to begin the commercial development of such species the functional stability of improved traits such as virus resistance expression must be determined.

The present report furthers our understanding of PTGS-based resistance to PPV by extending resistance analyses to a 4-year field trial. We know of no other study that has analyzed PTGS-based virus resistance in a temperate woody perennial. Such studies are critical if we are to utilize this technology for the improvement of these species.

This report shows that the hallmark characteristics of PTGS in C5 trees remain stable in the field. RNA blots (data not presented) and RT-PCR showed a reduction in the steady-state level of PPV-CP mRNA (Figure 2a). Methylation analyses indicated that the PPV-CP transgene was specifically methylated (Figure 3) while *gusA* and *npt*II transgenes were not. We also show that the promoter region of the resistant clone C5 is 4–10 times more methylated than the susceptible C3 clone. This may indicate a spread of

methylation from the CP gene to its promoter, at least into the extreme 3′ end adjacent to the PPV-CP gene. This spreading of methylation into the promoter region has been previously observed in a herbaceous system (Ingelbrecht et al., 1994). Spreading of methylation within the transcribed region but restricted to the transcribed region has also been observed (Sijen et al., 1996; Jones et al., 1999). The degree and pattern of methylation differs between the C3 and C5 clones. The degree of methylation is higher in C5 compared to C3 (11–24 times greater at the 340 *Sau*3AI site, and 13 times greater at the 660 *Sau*3AI site). When primers were designed that spanned both restriction sites (data not shown), no product was amplified from C3 while a product was clearly obtained from C5, suggesting that in the C5 clone both sites are concurrently methylated. A similar pattern of amplification/methylation was observed for the PPV-CP insert in C5 compared to C3 in an uninoculated field trial in the US (data not presented).

The current field trial illustrates the importance of the inoculation method for studying PPV resistance in the field. Graft- and aphid-inoculation differ in the levels of virus accumulation after 4 years in the field. Susceptible trees, both untransformed B70146 and PPV-CP transgenic C3, infected by CBI accumulated more virus than AVI trees (Figure 2c).

C5 trees left to AVI remained healthy throughout the study. AVI C5 trees never showed signs of infection and never tested positive for PPV (Figures 1 and 2). Further, controlled inoculations of C5 with viruliferous aphids in greenhouse and field tests have failed to infect C5 (data not presented), confirming the results of these field tests. In the early stage of field-testing (year two) a few leaves adjacent to each other were observed with PPV symptoms in CBI C5 trees. These leaves tested as positive for PPV by ELISA. Since that initial observation, very few leaves of these CBI C5 trees produced symptoms, they were generally ELISA and RT-PCR negative but a few samples were IC-RT-PCR positive (Figures 1e, f). Long term greenhouse studies have recently confirmed that graft-inoculated C5 trees can harbor a low level of PPV detectable only through IC-RT-PCR, with no symptoms, even when the infected rootstock was highly positive for PPV in terms of symptom expression and ELISA detection (http://www.intl-pag.org/pag/9/abstracts/W45_01.html). PPV ingress into the host by grafting and by aphid feeding occurs through different routes. Non-persistent aphid transmission generally deposits virus into epidermal cells. Virus replicates within the cell and then moves from cell-to-cell until the vascular tissue is reached, which serves as a conduit to all parts of the plant. Grafting an infected chip-bud allows a direct ingress of relatively high levels of virus directly into the vascular system of the host. If PTGS and its associated virus suppression is viewed as a competitive process then graft-inoculation that provides high titer and allows for ready systemic virus movement may favor virus suppression of PTGS at some level. Recent studies showed that the potyvirus helper component-proteinase (HC-pro) is an effective suppressor of silencing (Anandalakshmi et al., 1998), but it must be present at a high level (Brigneti et al., 1998).

Graft-inoculation is not the natural mode of infection. Introducing the virus through grafting could bypass the plant's natural defense mechanism. The influence of the mode of PPV transmission, the level of inoculum on long-term efficiency of PTGS and the determination of the location where PTGS takes place require further studies.

This report documents PTGS in providing high-level PPV resistance in the field under severe infection pressure. C5 trees exposed to natural aphid vectored infection after 4 years have not been infected, while almost all control plants became infected, illustrating the extremely high-level of resistance of this clone. Observations of this field trial after 6 years confirm these results with no AVI C5 trees infected and all control trees infected (data not presented). With levels of fruit quality and productivity suitable for the commercial market (unpublished), the C5 clone is potentially useful for plum production in areas of severe and endemic PPV. Further, results showing that the transgene insert and resistance are transferred to progeny of C5 as a single dominant trait make this clone useful as a source of PPV resistance that can be readily moved into new genotypes through traditional breeding (Ravelonandro et al., 1998b; Scorza et al., 1998).

The productive life of most fruit tree orchards is 10–25 years. The life of a particular clone can span decades or centuries in some cases. While results to date with C5 plum are promising, continued evaluation and the application of potential resistance-breaking treatments such as co-infection with multiple PPV strains and unrelated *Prunus* viruses will be necessary to truly verify the long-term stability of PTGS-based resistance in this temperate woody perennial crop.

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