# TRANSGENIC PLUMS EXPRESSING *PLUM POX VIRUS* COAT PROTEIN GENE DO NOT ASSIST THE DEVELOPMENT OF VIRUS RECOMBINANTS UNDER FIELD CONDITIONS

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## SUMMARY

The serological and genetic variability of *Plum pox virus* (PPV) isolates from transgenic plum trees expressing the PPV coat protein gene and conventional plums was analyzed. PPV isolates were characterized serologically by TAS-ELISA using PPV-D and PPV-M specific monoclonal antibodies and by molecular typing across three genomic regions as well as RFLP analysis of the 3' terminus of the CP gene. PCR products spanning the (Cter)CP and (Cter)NIb-(Nter)CP regions were sequenced. Sequence information revealed no significant difference between serological and molecular features of PPV isolates from transgenic and conventional plums that were growing together in experimental orchards for 6-8 years. These results show that transgenic plums do not promote the emergence of new PPV variants under field conditions.

*Key words*: pathogen derived resistance, GMO, CP gene, safety issue, recombinant viruses.

## INTRODUCTION

Pathogen derived resistance (PDR) (Sanford and Johnston, 1985) can provide an alternative to conventional breeding for obtaining virus-resistant plants. This technology holds great promise for perennial crops because conventional breeding is time-consuming and expensive (Dolgov and Hanke, 2006). In many cases, sources of virus resistance are unknown, multigenic, or exist only in primary germplasm, requiring many generations of selection to produce resistant commercial cultivars (Hartmann and Petruschke, 2002). In the case of *Plum pox virus* (PPV), the most devastating virus of stone fruits (Cambra *et al.*, 2006) PDR has been used to produce plums resistant to PPV (Ravelonandro *et al.*, 1997; Malinowski *et al.*, 2006). The mechanism of resistance in PPV-resistant plums has been shown to be post-transcriptional gene silencing (PTGS) in which both transgene mRNA and homologous viral RNA are degraded (Scorza *et al.*, 2001; Hily *et al.*, 2004).

Several environmental safety issues have been raised with the application of PDR, particularly when viral transgene transcripts are expressed. The issues of concern include complementation, transcapsidation, synergism, and recombination (Thomas *et al.*, 1998; Tepfer, 2002). Recombination is of particular concern since it has been suggested that a virus transgene inserted in a plant genome may generate recombinant viruses with new biological proprieties (Wintermantel and Schoelz, 1996). Most studies of recombination have been carried out under greenhouse conditions and under high to moderate selection pressure to favor the development of recombinants (Tepfer, 2002; Turturo *et al.*, 2008), including PPV (Varrelmann *et al.*, 2000). Perennial crops are grown in the field for many years and are continually exposed to virus infection. Therefore, perennial crops engineered for virus resistance may have increased potential for recombination compared to annual crops. Yet few long-term studies have been undertaken with perennial crops to address the emergence of recombinant viruses (Vigne *et al.*, 2004; Fuchs and Gonsalves, 2007; Capote *et al.*, 2008).

Recombination has been assessed in PPV CP transgenic and conventional plums grown in the field under Mediterranean conditions. PPV-D strain was used as inoculum but no recombination between transgene transcript and viral RNA was detected, nor was the genetic diversity of virus populations affected (Capote *et al.*, 2008). Here, we expanded on this work and evaluated the occurrence of recombination in PPV CP transgenic plum trees that were exposed to infection by the D and Rec strains of PPV for 6-8 years in the field under continental European conditions.

## MATERIALS AND METHODS

**Plum trees and field plots**. The transgenic plum clones C2, C3, C4 and PT3 planted for these trials express the CP gene (Scorza *et al.*, 1994). The transgenic clone C6 contains one copy of the PPV CP gene and

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does not accumulate viral CP (Scorza *et al.*, 1994). All these transgenic clones are susceptible to PPV (Ravelonandro *et al.*, 1997; Hily *et al.*, 2004; Malinowski *et al.*, 2006). Transgenic clones were grafted onto the GF-8.1 rootstock (*Prunus mariana)* at INRA in Bordeaux (France), and subsequently established in experimental orchards at the Fruit Research and Development Station Bistrita, Romania under Ministry of Agriculture import authorization no. 1166/02/1996. Following EU recommendations, the experimental plots were surrounded by a buffer of apple trees. The first plot was set up in 1996 with 55 transgenic plum trees belonging to five clones (C2, C3, C4, C6 and PT3). Ten trees each of transgenic clones C2, C3, C4, and C6, and five of transgenic clone PT3 were planted in the spring. Plum trees were planted within a 6-year-old PPV-infected plum orchard in a single row, at a spacing of 4 m between trees and 5 m between rows. In this plot, a high number of transgenic plants died after planting, probably due to the poor adaptation of the GF-8.1 rootstock to the heavy soil and environmental conditions in the Carpatian region in Romania. In order to improve tree survival, a new experimental plot was established in 1998 with 11 plum trees belonging to four transgenic clones (C2- three trees, C4- three trees, C6- two trees, and PT3- three trees) grafted onto myrobolan rootstock. Tree spacing was identical as in 1996 but the transgenic plums were randomly dispersed within the orchard.

**PPV infection and sampling.** Leaf samples were collected from transgenic trees showing typical PPV symptoms and symptomatic conventional trees surrounding transgenic trees in June 2004 and/or 2005. Leaf samples were collected at random throughout the canopy. If symptoms were limited to particular branches, leaves were only sampled from symptomatic branches. Virus infection was confirmed by serological and molecular detection.

**Serological and molecular detection of PPV**. Serological tests were performed by DAS-ELISA (Clark and Adams, 1977) using a commercial polyclonal antiserum to PPV according to the manufacturer's instructions (Bioreba, Switzerland). Absorbance values were measured at 405 nm after 1 h substrate hydrolysis. Samples were considered positive if their absorbance values were more than twice those of the negative control. Molecular PPV detection was performed by IC-RT-PCR using primer pair P1/P2 that amplifies a 243 bp fragment corresponding to the 3'-terminus of the PPV CP gene (Wetzel *et al.*, 1991). For immunocapture, PPV was trapped with the above PPV polyclonal antiserum adsorbed on an Eppendorf microtube. The enhanced Avian kit (Sigma, USA) was used for RT-PCR. The thermal cycling scheme consisted of 30 min at 50°C followed by 2 min at 94°C and 35 cycles of 30 sec at 94°C, 45 sec at 61°C and 60 sec at 72°C and a final elongation step of 10 min at 72°C. An aliquot of the amplified products (10 µl) was fractionated in 1.5% agarose gel electrophoresis in Tris-borate EDTA (TBE) buffer. DNA bands were visualized by ethidium bromide staining under UV light.

**Strain characterization.** In order to identify the serotype of the PPV isolates, TAS - ELISA was run with specific monoclonal antibodies raised to PPV-D (Dideron or chlorotic strain) (Durviz, Spain) (Cambra *et al.*, 2004) and PPV-M (Marcus or necrotic strain) (Agritest, Italy) (Boscia *et al.*, 1997).

Molecular strain typing was done by IC-RT-PCR targeting three genomic regions corresponding to: (i) (Cter) CP using specific primers PD and PM that distinguish the two major PPV strains D and M (Olmos *et al.*, 1997); (ii) (Cter) NIb - (Nter)CP using the primer pair mD5/mM3 (Subr *et al.*, 2004) that detects recombinant PPV strain (PPV-Rec) between D and M (Glasa *et al.*, 2002, 2004); (iii) CI using CIf/ CID or CIM primer sets (Glasa *et al.*, 2002) to confirm the presence of PPV-Rec. Aliquots of PCR products corresponding to (Cter) CP were subjected to RFLP analysis in order to distinguish the D and M strains based on *Rsa* I polymorphism. Digested products were fractionated in 8% polyacrylamide gel electrophoresis (PAGE) in TBE buffer and photographed under UV light after staining with ethidium bromide.

**Sequencing.** To confirm the molecular variability of PPV isolates, amplified DNA products were purified by Wizard SV Gel and PCR Clean-Up System (Promega, USA) and sequenced by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on the ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA). Alignment of nucleotide sequences from 44 PCR products corresponding to the (Cter)CP gene (15 PPV isolates from transgenic plums and 29 PPV isolates from conventional plums) and seven amplified fragments spanning the (Cter) NIb - (Nter)CP region (four isolates from transgenic plums and three isolates from conventional plums) was done using the program package BioEdit version 5.0.9 (Hall, 1999). Subsequently, the newly sequenced fragments were submitted to GenBank and compared with other PPV sequences available in this database. A phylogenetic tree was constructed with the Mega 3.1 program using Minimum Evolution method Jukes-Cantor model (Bootstrap value 10,000) for sequences corresponding to the (Cter)CP gene (Felsenstein, 2004).

## RESULTS

**Variability of PPV populations.** PPV isolates D and

Rec are endemic in Bistrita (Romania) and natural aphid populations readily transmit PPV isolates among plum trees in experimental orchards (Zagrai *et al.,* 2008). The first trial with transgenic plums was established in 1996 in an experimental orchard for which 23% (49 of 211) of the trees were infected with PPV. In that plot, 10 transgenic and 37 conventional plums showing typical PPV symptoms were sampled in 2004 and 2005. The second trial with transgenic plums was established in 1998 in another experimental orchard for which 4.5% (27 of 598) of the trees were infected with PPV. In the second plot, five transgenic and 17 symptomatic conventional plums were sampled.

TAS-ELISA indicated that the 15 isolates from transgenic plums were PPV-D (eight isolates) and PPV-M (seven isolates). These results were confirmed by IC-RT-PCR using PD and PM specific primers, respectively. Similarly, the two PPV strains were identified by RFLP analysis and the presence of *Rsa* I sites in PPV-D strain (Table 1). All PPV isolates selected from conventional plums surrounding the transgenic plums also reacted positively to at least one of the two monoclonal antibodies as well as to PPV-D or/and PPV-M specific primers (Table 2). A slight discrepancy between serological and molecular test results was observed. Indeed, 28 of 54 isolates were identified as PPV-D by TAS-ELISA, 24 of

**Table 1.** Serological and molecular differentiation of PPV isolates from transgenic plums.

| Isolate (row/tree) | DAS / TAS-ELISA | | | | IC-RT-PCR | | | | RFLP *Rsa*I | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | PPV poly | PPV-D | PPV-M | PPV-D+M | PPV poly | PPV-D | PPV-M | PPV-D+M | PPV-D | PPV-M | PPV-D+M |
| *Plot no. 1* | | | | | | | | | | | |
| C6-2 (3/21) | + | + | - | - | + | + | - | - | + | - | - |
| C6-2 (3/23) | + | + | - | - | + | + | - | - | + | - | - |
| PT3-2 (3/25) | + | + | - | - | + | + | - | - | + | - | - |
| C6-2 (3/22) | + | + | - | - | + | + | - | - | + | - | - |
| C3-9 (3/6) | + | - | + | - | + | - | + | - | - | + | - |
| C3-9 (3/8) | + | - | + | - | + | - | + | - | - | + | - |
| C3-9 (3/9) | + | - | + | - | + | - | + | - | - | + | - |
| C3-9 (3/10) | + | + | - | - | + | + | - | - | + | - | - |
| C4-6 (3/15) | + | + | - | - | + | + | - | - | + | - | - |
| C2-10 (3/2) | + | - | + | - | + | - | + | - | - | + | - |
| *Plot no. 2* | | | | | | | | | | | |
| PT3-2 (9/11) | + | + | - | - | + | + | - | - | + | - | - |
| PT3-2 (9/12) | + | + | - | - | + | + | - | - | + | - | - |
| C4-6 (9/21) | + | - | + | - | + | - | + | - | - | + | - |
| C6-2 (15/20) | + | - | + | - | + | - | + | - | - | + | - |
| C2-10 (16/36) | + | - | + | - | + | - | + | - | - | + | - |
| TOTAL (%) | 15 (100) | 8 (53.3) | 7 (46.7) | 0 (0) | 15 (100,0) | 8 (53.3) | 7 (46.7) | 0 (0 ) | 8 (53.3) | 7 (46.7) | 0 (0 ) |

**Table 2.** Serological and molecular differentiation of PPV isolates from conventional plums surrounding transgenic plums.

| No. of isolates | DAS / TAS-ELISA | | | | IC-RT-PCR | | | | RFLP *Rsa* I | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | PPV poly | PPV-D | PPV-M | PPV-D+M | PPV poly | PPV-D | PPV-M | PPV-D+M | PPV-D | PPV-M | PPV-D+M |
| *Plot no. 1* | | | | | | | | | | | |
| 24 | + | + | - | - | + | + | - | - | + | - | - |
| 10 | + | - | + | - | + | - | + | - | - | + | - |
| 1 | + | + | - | - | + | - | - | + | - | - | + |
| 1 | + | - | + | - | + | - | - | + | - | - | + |
| 1 | + | - | - | + | + | - | - | + | - | - | + |
| *Plot no. 2* | | | | | | | | | | | |
| 3 | + | + | - | - | + | + | - | - | + | - | - |
| 13 | + | - | + | - | + | - | + | - | - | + | - |
| 1 | + | - | - | + | + | + | - | - | + | - | - |
| TOTAL (%) | 54 (100) | 28 (51.9) | 24 (44.4) | 2 (3.7) | 54 (100) | 28 (51.9) | 23 (42.6) | 3 (5.5) | 28 (51.9) | 23 (42.6) | 3 (5.5) |

**Table 3.** Molecular typing of PPV isolates from transgenic and conventional plums in different viral genomic regions.

| Plot no. | Number of isolates | | Target region | | |
|---|---|---|---|---|---|
| | Transgenic | Conventional | (C-ter) CP | C-ter (NIb)–(N ter) CP | CI |
| 1 | 6 | 24 | D | - | D |
| | 4 | 10 | M | Rec | D |
| | 0 | 3 | D+M | Rec | D |
| 2 | 2 | 4 | D | - | D |
| | 3 | 13 | M | Rec | D |
| | 0 | 0 | D+M | Rec | D |
| Total | 8 | 28 | D | - | D |
| | 7 | 23 | M | Rec | D |
| | 0 | 3 | D+M | Rec | D |

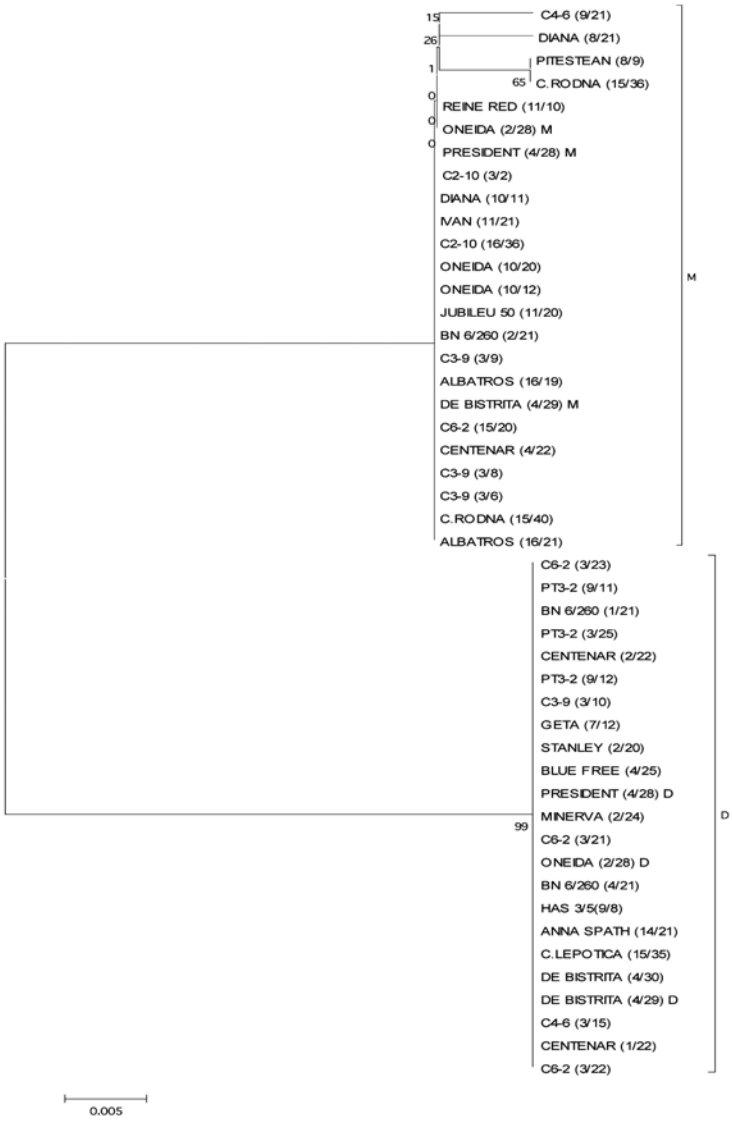


**Fig. 1.** Phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to the 3'-terminus of the PPV coat protein gene. Isolates President 4/28, Oneida 2/28 and De Bistrita 4/29 represent mixed infection of PPV D and M strains.

54 as PPV-M and two as a mixed infection of PPV-D and PPV-M. IC-RT-PCR analysis revealed the presence of PPV-D in 28 isolates, PPV-M in 23 isolates, and a mixed infection of D and M strains in three isolates. These results were confirmed by RFLP analysis using *Rsa*I digestion.

**Occurrence of natural PPV-Rec.** All PPV isolates from transgenic and conventional plums initially typed as PPV-M in the (Cter)CP region were identified as PPV-Rec in the (Cter) NIb - (Nter)CP region by IC-RT-PCR using primer pair mD5/mM3 (Subr *et al.*, 2004) (Table 3). As expected no serological differentiation was observed between PPV-M and PPV-Rec. In addition, the use of specific primers to distinguish strains D and M in the CI region detected only fragments of PPV-D, confirming their status as PPV-Rec.

The distribution of PPV strains in transgenic (PPV-D: 53% and PPV-Rec: 47%) and conventional (PPV-D: 52% and PPV-Rec: 43%) plums surrounding the transgenic plums was similar, except for mixed infections (PPV-D + PPV-Rec: 5.5%) that were detected only in conventional plums. Mixed PPV-D and -Rec infections in conventional plums may have been the result of older trees exposed over longer periods of time to aphids carrying both strains.

**No emergence of new PPV variants in transgenic plums.** The phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to the 3'-terminus of the PPV CP gene confirmed two major groups and a similarity of PPV isolates from transgenic and conventional plums (Fig. 1). No recombination was found in this genomic region. Our PPV-D and -Rec sequences were 100% and 98-99% identical to the sequences from the NCBI database, respectively. Four nucleotide substitutions were detected in PPV-Rec, one in an isolate from a transgenic plum (C4-6, row 9/tree 21) and three in isolates from conventional plums [Pitestean (8/9), C. Rodna (15/36), Diana (8/21)]. The PPV sequences determined in this study are available in Gen-

Bank as accessions numbers HQ218871 to HQ218917.

To check if the recombination breakpoint position suspected to occur in the (C-ter)NIb - (N-ter)CP region corresponded with those of PPV-Rec previously reported in conventional plums (Glasa *et al.*, 2002, 2004), seven PCR products spanning this genomic region [four from transgenic plums (accession Nos HQ218918-HQ218921) and three from conventional plums (accession Nos HQ218922-HQ218924)], were sequenced (Fig. 2). The multiple nucleotide sequence alignment indicated that the recombination breakpoint is located at the same nucleotide position 8450 as other PPV-Rec. These results were consistent with the notion that the genetic variability of PPV-Rec isolates is similar in transgenic and conventional plums. In addition, a high similarity (98-99%) with different sequences of PPV -Rec previously reported [BNE-10 (accession No. AF450311), LOZ-3 (accession No. AF450312), BOR-3 (accession No. AY028309)] was found. This genetic similarity confirms that PPV Rec is widespread in Romania and likely belongs to the same ancestral group with a common evolutionary origin (Glasa *et al.*, 2004; Zagrai *et al.*, 2008).

## DISCUSSION

In this report we show that transgenic plum-trees expressing a PPV CP gene do not assist the emergence of PPV recombinants in the field under continental European conditions. The transgenic plums that are PPV susceptible and produce PPV-CP mRNA (except for the C-6 clone) were grown for 6-8 years in experimental orchards, and no significant differences in serological and genetic variability of PPV isolates sampled in transgenic plums and surrounding conventional plum trees was detected. As previously observed by Capote *et al.* (2008), there are no apparent differences in the genetic make up of PPV isolates from transgenic and conventional plum trees. The similarity in the ratio between

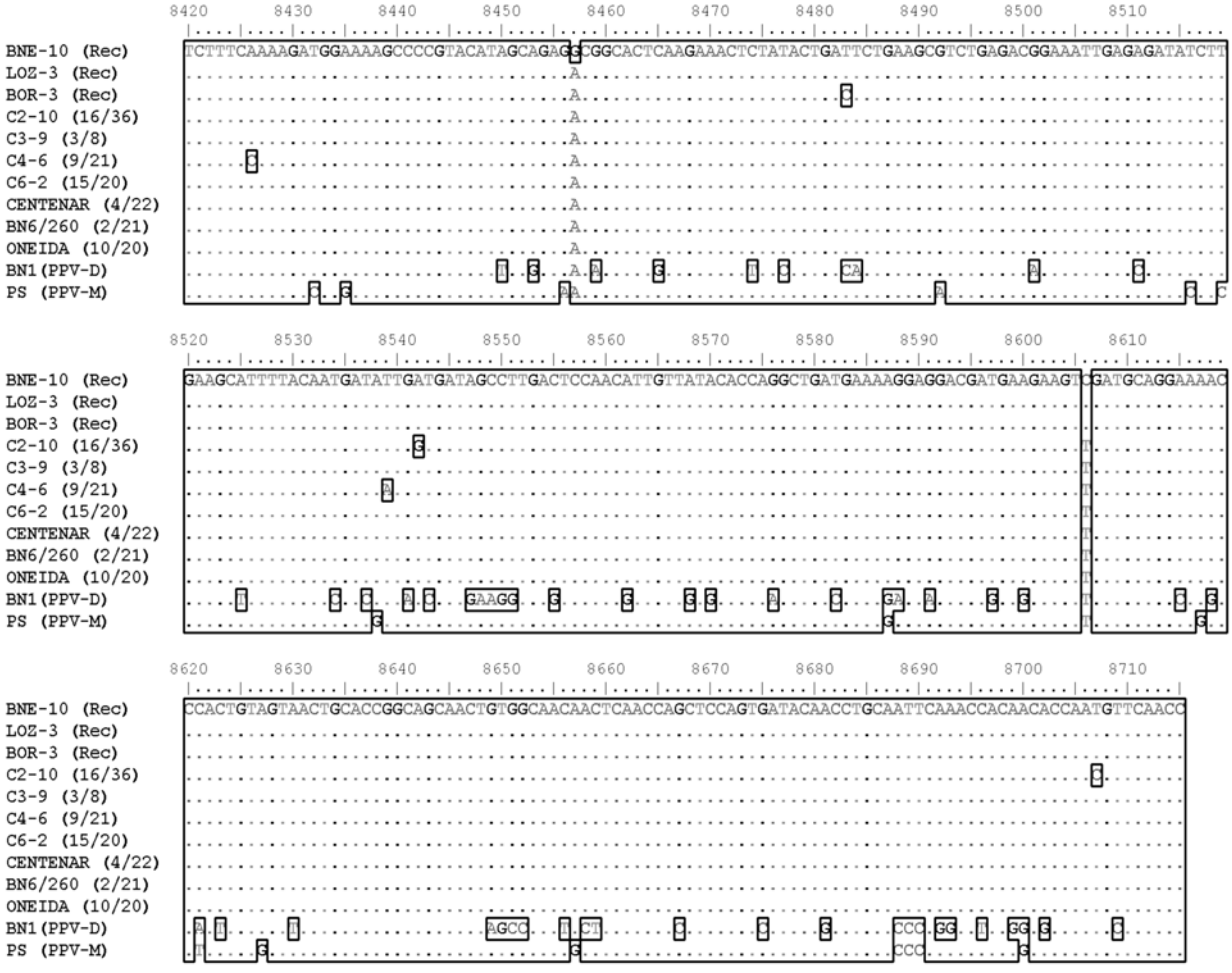


**Fig. 2.** Multiple alignment of recombinant sequences (NIb/CP) of seven Romanian PPV isolates from transgenic [C2-10 (16/36), C3-9 (3/8), C4-6 (9/21), C6-2 (15/20)] and conventional plums [Centenar (4/22), BN 6/260 (2/21), Oneida (10/20)] and three isolates [BNE-10 (accession number AF450311), LOZ-3 (accession number AF450312), BOR–3 (accession number AY028309)] previously reported.

PPV strains in transgenic and conventional trees suggests that aphid vectors do not differentiate between the two types of plums. The occurrence of a few nucleotide mutations (substitutions and silent mutations) was identified in the CP gene of a few isolates. These results were consistent with the investigation of PPV D populations in Spain (Capote *et al*., 2008). The major difference between our study and the work of Capote *et al.* (2008) is the diversity of PPV populations. Trees were exposed to PPV D and Rec strains in Romania but only to D strains in Spain. The fact that identical transgenic plum trees were evaluated under distinct environmental conditions for which diverse aphid populations and climatic factors occur in continental Romania and Mediterranean Spain strengthen our collective conclusions on a lack of effect of transgenic trees on the serological and genetic variability of natural PPV populations.

The fact that new PPV variants were not detected in transgenic plums is in contrast with observations in annual species where the emergence of recombinants is well documented (Tefper, 2002; Turturo *et al*., 2008). Although PPV Rec isolates were identified in our study, the recombination breakpoint was identical in the (C-ter)NIb–(N-ter)CP region of isolates from transgenic and conventional plums, indicating that recombination did not involve transgene PPV CP transcripts. Instead, the presence of PPV Rec isolates in transgenic plums resulted from aphid-mediated transmission from adjacent naturally infected nontransgenic plum trees.

Our results and those of Capote *et al.* (2008) indicate that transgene transcripts in plum trees expressing a PPV CP gene do not contribute to the creation of PPV recombinants with PPV D and Rec strains. Further, since PPV is the only known potyvirus transmitted in a non-persistent manner in *Prunus*, the risk of heteroencapsidation is limited unlike in annual crops for which multiple potyviruses can coexist and interact with aphid vectors (Fuchs and Gonsalves, 2007).

Together with results reported from work with transgenic grapevines (Vigne *et al*., 2004), our findings provide strong evidence that transgenic perennial fruit trees expressing a virus CP gene do not assist the development of recombinants under field conditions. Field studies in woody perennial hosts do not support recombination reported from greenhouse studies (Tepfer, 2002; Turturo *et al.,* 2008).

A high level and a durable resistance to PPV was achieved in transgenic plums through PTGS (Scorza *et al*., 2001; Hily *et al*., 2004) and the production of siRNA (Hily *et al*., 2005; Scorza *et al*., 2007; Kundu *et al*., 2008). The resistant transgenic clone C-5 does not produce detectable PPV-CP mRNA (Scorza *et al*., 1994) but accumulates the siRNA doublet that has been analyzed extensively by Hily *et al.* (2005) and Kundu *et al.* (2008). C-5 plum trees were not evaluated in this study since this clone is not infected following natural aphid-mediated transmission of PPV (Malinowski *et al*., 2006).

In summary, our study shows that recombination was not detected in PPV-susceptible transgenic plums expressing the PPV CP gene. Also the genetic diversity of PPV strains in transgenic and conventional plums was similar. Therefore, we conclude that transgenic plums expressing the PPV CP gene do not present a greater risk in terms of virus recombination and diversity than conventional plums.

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