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Overview of the Investigations of Transgenic Plums in Romania

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Abstract. Transgenic plums of *Prunus domestica* L. transformed with the *Plum pox* virus coat protein gene (PPV-CP) were the subjects of three experiments undertaken in Romania. In the first experiment PPV-CP transgenic clones C2, C3, C4, C5, C6 and PT3 were evaluated for Sharka resistance under high natural infection pressure. Transgenic clone C5, subsequently named 'HoneySweet', showed high resistance to PPV. None of the C5 trees became naturally infected by aphids for more than ten years. The resistance of C5 was based on post-transcriptional gene silencing (PTGS). In the second experiment we assessed the effect of two heterologous viruses (*Prunus necrotic ringspot* virus and *Prune dwarf* virus) on the efficacy and stability of PTGS - mediated resistance to PPV displayed by the C5 plum. The engineered resistance to PPV in C5 transgenic plums was stable and was not suppressed by the presence of the assayed heterologous viruses. Some PPV-CP transgenic plum clones that are susceptible to PPV including C2, C3, C4 and PT3 display a constitutive transcription of PPV-CP sequence. In the third experiment we used these plants to assess the environmental safety issues related to potential hazards concerning the emergence of PPV variants. The serological and molecular variability of PPV detected in transgenic and in conventional plums revealed that the transgenic plums do not affect the diversity of indigenous PPV populations. We suggest that the safety and efficacy data developed in Romania and other European countries and in the United States of America over the last 15 years justifies an expansion of the field tests in Romania and in other countries that are experiencing the damages to plum production caused by PPV.

Keywords: *Plum pox* virus, transgenic plum, CP gene, resistance, safety issue

INTRODUCTION

Plum pox virus (PPV) is the causal agent of the most devastating Sharka diseases of *Prunus* species, leading to important economic losses (Cambra *et al*., 2006). Due to the rapid spread of PPV by aphids and the presence of many potential hosts, Sharka disease is difficult to eradicate once it has become established in an area. It is known that the use of resistant cultivars represents the most efficient solution to control PPV infection.

Conventional breeding has exploited naturally occurring resistance. Unfortunately, the paucity of natural resistance genes has hampered the efforts to control Sharka disease. However, a multigenic hypersensitive reaction has been reported in *Prunus domestica* and a resistant hypersensitive cultivar "Jojo" has been released (Hartman and Petruschke, 2002). The absolute resistance of this cultivar was refuted by Polak *et al*. (2005) which showed that PPV can be present in the tissue of Jojo cv. because the virus was transferred to the rootstock.

The utilization of natural sources of resistance is important for the development of new varieties but it is difficult and long-term to incorporate such resistance into new stone fruits varieties through conventional breeding. Genetic engineering offers the opportunity to

develop resistant plums by introducing a virus gene segment into the DNA of *Prunus* host plants. Following the strategy of Pathogen Derived Resistance (PDR) – (Sanford and Johnson, 1985) as a complementary approach to conventional breeding, transgenic European plums (*Prunus domestica* L.) containing the CP gene of PPV were developed (Scorza *et. al.*, 1994). One transgenic line, C5, subsequently named 'HoneySweet' (Scorza *et al.*, 2007) was found to be highly resistant to graft- and aphid-mediated inoculation by PPV in greenhouse (Ravelonandro *et al.*, 1997, 2000; Hily *et al.*, 2004.). Field trials have been carried out in Poland, Spain (Malinowski *et al.*, 2006) and Romania (Zagrai *et al.*, 2008a) verifying that PDR evaluated in two different environments (continental and Mediterranean) was durable.

C5 viral resistance is based on Post Transcriptional Gene Silencing (PTGS) (Scorza *et al.*, 2001). As a response to this defence mechanism, many viruses encode gene-silencing suppressor proteins acting at different points in the PTGS pathway (Anandalakshmi *et al.*, 1998; Voinnet, 2001). While viral suppression of gene silencing has been demonstrated in herbaceous species, it has not been reported in most crops including woody plant species or/and under field conditions.

Because PPV-CP sequences are constitutively expressed in transgenic plums, the potential of the emergence of a PPV variant was suggested. As reported by Wintermantel and Schoelz, (1996), the virus transgene inserted in plant genome may generate new recombinant viruses with new biological proprieties. Interestingly, this scenario has been only reported with transgenic herbaceous plants in greenhouse conditions (Tepfer, 2002). Few studies of recombination with agronomically important virus-resistant transgenic crops have been published. Vigne *et al.* (2004) reported that in field tests virus recombination in transgenic grape expressing *Grapevine fanleaf* virus CP did not occur.

This paper is an overview of the results of three experiments performed at Fruit Research & Development Station Bistrita, Romania: the first was focused to field resistance trials of several transgenic plum clones (Zagrai *et al.*, 2008a), the second was addressed to the stability of RNA silencing displayed by the C5 clone under the coexistence of PPV and two heterologous ilarviruses (Zagrai *et al.*, 2008b), and the third was aimed to the environmental safety issue related to transgenic plums expressing PPV-CP gene (Zagrai *et al.*, 2010).

MATERIAL AND METHODS

Field resistance trials

The studies were carried out under conditions of high PPV inoculum pressure in three field experimental plots located at Fruit Research and Development Station Bistrita, Romania.

The first plot was set up in 1996 with 60 transgenic plum trees belonging to C2, C3, C4, C5, C6, PT3 clones. These plum trees were planted inside a 6 year old orchard, in a single row under high natural PPV infection pressure (23% of the conventional plums were infected).

The second experimental plot included only the C5 transgenic clone (8 trees) planted in 1998 in the vicinity of young conventional plums that are known to be tolerant to PPV. A large number of infected conventional plum trees which flanked the experimental plot provided a high infection pressure.

The third plot was set up in 1998 when 21 plum trees belonging to five transgenic clones (C2- three trees, C4- three, C5- ten, C6- two, and PT3- three trees) were planted in a new orchard within young trees (two years old) where PPV infection was rated to 4.5% of infected trees. In this plot the transgenic plums were randomly dispersed within the orchard.

The monitoring of disease based on visual observations of PPV symptoms on leaves

and by serological and molecular diagnosis. Serological tests were performed by Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA) – Clark and Adams (1977), using polyclonal antibodies according to the manufacturer (Bioreba). Molecular detection was made by Immunocapture-Reverse Transcription-Polymerase Chain Reaction (IC/RT-PCR) using the P1/P2 polyvalent primer pairs (Wetzel *et al.*, 1991).

Field trials assessment of the stability of RNA silencing in C5 clone under artificial mixed infections of PPV and heterologous viruses

Two experiments were initiated in the field, in an experimental orchard and in a nursery respectively, and another one in the greenhouse. Challenge heterologous viruses were *Prunus necrotic ring spot* virus (PNRSV) and *Prune dwarf* virus (PDV). The C5 transgenic clone was chip-bud inoculated with PPV (D strain) and with the combinations PPV + PDV or PPV + PNRSV.

Six C5 trees were subjected to chip-bud graft inoculation in experimental orchard. Two C5 plum trees were used for each virus combination. Half of the branches of each tree were graft inoculated and the other half not.

In experimental nursery, the inoculation with the viruses was made via rootstock as follow: buds of transgenic or conventional (control) plums were inserted on the top of grafting area, buds with PPV were inserted in the middle and buds with heterologous viruses were inserted at the bottom. Ten plants were used for each virus combination.

The graft inoculations were performed under greenhouse conditions as in the nursery. Five plants were inoculated for each virus combination.

For each experiment, conventional plants were similar inoculated and used as controls.

Virus infection (PPV and heterologous viruses) was recorded in both experiments by visual monitoring of symptom development and by serological (DAS-ELISA) and molecular (IC-RT-PCR) methods.

Environmental safety assessment related to transgenic plums expressing PPV-CP gene

The infected transgenic trees and symptomatic conventional trees surrounding each sampled transgenic tree were considered as PPV isolate sources. All PPV isolates identified in transgenic plums, 15 isolates respectively and 54 isolates selected from conventional plums surrounding the transgenic plums were serological and molecular analyzed.

Serological differentiation was made by TAS (Triple Antibody Sandwich) -ELISA with the PPV-D (Cambra *et al.*, 2004) and PPV-M (Boscia *et. al.*, 1997) specific monoclonal antibodies.

Molecular strain typing was done by IC/-RT-PCR targeting the genomic regions corresponding to (Cter) CP, (Cter) NIb – (Nter)CP, and CI. The molecular variability was confirmed by sequencing of 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).

RESULTS AND DISCUSSION

Field resistance trials

In the first plot, after more than ten years of field testing, 11 transgenic plum trees belonging to C2, C3, C4, C6 and PT3 clones were found infected (44% of trees) while the percentage of conventional plums infected increased from 23% in 1996 to about 76% in 2006. Although the presence of aphid vectors was often observed on C5, this transgenic clone

remained uninfected exhibiting a high and durable resistance to PPV. The temporal spread of PPV in the first plot showed a continual increase in infection of conventional plums and a delay of infection in C2, C3, and C4.

In the second plot none of the C5 transgenic trees were infected by PPV during eight years of field testing while most of the tolerant conventional plums surrounding C5 become infected. The temporal spread of PPV in the second plot revealed also a continual increase of infection in conventional plums while C5 remained PPV free.

In the third plot, after five years of field testing only C5 remained uninfected. The infection in conventional plums increased from 4.5% in 1998 to 39.8% in 2003. This demonstrated an exposure to high infection pressure mediated by aphid vectors. The temporal spread of PPV in the third plot showed that, after five years of field testing the infection rate in conventional and transgenic plums (excepted C5) was similar.

Overall, no C5 trees became infected. Under these conditions, this clone shows a high and durable resistance to natural PPV infection.

Field trials assessment of the stability of RNA silencing in C5 clone under artificial mixed infections of PPV and heterologous viruses

Regardless of singular (PPV) or mixed (PPV+PDV, PPV+PNRSV) infection, C5 transgenic plums revealed a similar behavior in regard to PPV infection. PPV was trans located from the inoculum to the C5 plants and could produce a limited infection independently of the presence of the heterologous viruses. In all three experiments, PPV could be observed and detected by DAS-ELISA and IC-RT-PCR only near the inoculum points. In addition, the intensity of PPV symptoms (discrete diffuse spots) in this part of the plants were extremely mild, and the viral concentration was generally very low. Across all experiments (orchard, nursery, greenhouse), the infection remained close to the inoculation site and did not systemically spread, indicating an effective inhibition of the virus replication. Conversely, very severe PPV symptoms and high viral concentration was obtained in the inoculated conventional plums which became systematically infected by PPV.

No differences in symptoms development or PPV spread was observed on C5 when PPV was inoculated alone or in combination with PDV or PNRSV. The inoculation of C5 transgenic plums with PPV and the two ilarviruses (PNRSV and PDV) in the field and in the greenhouse did not affect the efficacy and stability of PTGS. As a consequence, resistance to PPV did not break down. Malinowski *et al.* (2006) showed a similar situation in C5 trees graft-inoculated with PPV in an experimental open-field trial in Poland. Our results showed that there is no evidence that heterologous viruses can affect the stability of the engineered protection in transgenic C5 plums.

Environmental safety assessment related to transgenic plums expressing PPV-CP gene

The serological and molecular differentiation revealed a similar distribution of PPV strains in transgenic and conventional plums surrounding the transgenic plums (transgenic plum: PPV-D – 53 %, PPV-Rec. – 47 %; conventional plum: PPV-D – 52 %, PPV-Rec. – 43 %, PPV-D + PPV-Rec.– 5 %). Mixed 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. This similarity can suggest that aphid vectors do not make any differentiation between plums tested.

The phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to C-terminus of PPV coat protein confirmed the similarity of PPV isolates from transgenic and conventional plums. Also, in the case of PPV-Rec isolates, the multiple nucleotide sequence alignment corresponding to (Cter) NIb – (Nter)CP indicated that the

recombination breakpoint is located at the same nucleotide position as other PPV-Rec, and a high similarity with different sequences of PPV -Rec previously reported (Glasa *et al.*, 2002) was found.

It should be stressed that the transgenic resistant C-5 plum could not be sampled in this study since this clone cannot be infected by natural aphid transmission due to its high and durable resistance to natural PPV infection. It is therefore considered to be particularly safe in the sense of not affecting PPV strain variability or recombination.

Our results and those of Capote *et al.* (2008) indicate that transgenic plums expressing the PPV CP gene do not present a greater risk in terms of virus recombination and diversity than conventional plums.

Overall, the data developed in Romania, and other European countries (France, Poland, Spain, Czech Republic) and in the United States of America over the last 15 years demonstrated both the advantages of transgenic plum to control the spread of PPV and the lack of negative environmental impacts. Therefore, we suggest that these safety and efficacy data justifies an expansion of the field tests in Romania and in other countries that are experiencing the damages to plum production caused by PPV.

CONCLUSIONS

Field resistance trials performed in Romania confirmed a high and durable resistance to natural PPV infection of C5 transgenic plum.

Mixed infection comprising PPV and *Prunus necrotic ringspot* or *Prune dwarf* ilarviruses did not affect the efficacy and stability of PTGS in C5 transgenic plum.

Transgenic plums expressing PPV-CP gene do not represent an environmental risk for any emerging PPV strain.

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