LETTER TO THE EDITOR

SAFETY ASSESSMENT OF TRANSGENIC PLUMS AND GRAPEVINES EXPRESSING VIRAL COAT PROTEIN GENES: NEW INSIGHTS INTO REAL ENVIRONMENTAL IMPACT OF PERENNIAL PLANTS ENGINEERED FOR VIRUS RESISTANCE

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SUMMARY

The potential impact of transgenic plums and grapevines expressing viral coat protein (CP) gene constructs on the diversity and dynamics of virus populations was assessed under open and confined conditions in the frame of a research program sponsored by the European Commission. Across all field trials conducted in different locations (France, Romania, and Spain) and environments (continental and Mediterranean), transgenic plums expressing the CP gene of *Plum pox virus* (PPV) and transgenic grapevines expressing the CP gene of *Grapevine fanleaf virus* (GFLV) had no detectable effect on the emergence of recombinant PPV and GFLV species over eight-ten and three years, respectively. Also, no statistically significant difference was found in the number and type of aphids, including viruliferous individuals, and other arthropods that visited transgenic and nontransgenic plum trees. In addition, *Apple chlorotic leaf spot virus*, *Prune dwarf virus*, and *Prunus necrotic ringspot virus* did not influence the stability of the engineered resistance to PPV in co-infected transgenic plums over three dormancy periods. Further, under confined conditions, no recombinant virus was found to detectable level in transgenic grapevines expressing the CP gene of *Grapevine virus A* (GVA) or *Grapevine virus B* (GVB) that were challenged with the homologous or heterologous virus, despite high accumulation of transgene transcripts. Also, translocation of transgene-derived products, i.e. protein, mRNAs and siRNAs, did not occur to detectable level from transgenic grapevine rootstocks expressing the GFLV CP gene to nontransgenic scions. Altogether, our transgenic plums and grapevines expressing viral genes had a neutral impact on virus populations and non-target organisms over extended time. These findings provide new insights into the environmental impact of transgenic perennial crops engineered for virus resistance. It is expected that they will assist national and international regulatory authorities in making scientifically based decisions for the release of virus-resistant transgenic crops.

Key words: Transgenic plants, genetic engineering, recombinant DNA, risk assessment, coat protein gene, PPV, GFLV, GVA, GVB.

INTRODUCTION

The application of the concept of pathogen-derived resistance (Sanford and Johnston, 1985) opened new horizons for the development of virus-resistant plants. Pathogen-derived resistance, in which resistance to a virus is engineered in transgenic plants through the expression of a segment of the virus genome, has been applied successfully against numerous viruses (Tepfer, 2002). Engineered virus resistance was first described with *Tobacco mosaic virus* (TMV) in transgenic *Nicotiana tabacum* cv. Xanthi expressing the TMV coat protein

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(CP) gene (Powel Abel *et al.*, 1986). Twenty years after its discovery, pathogen-derived resistance has lead to the creation of many virus-resistant transgenic plants, some of which, including squash, papaya, pepper, and tomato, have been commercially released in the United States of America (Shankula *et al.*, 2005) and the People's Republic of China (Huang *et al.*, 2002). It has been shown that the antiviral pathways of RNA silencing confer resistance to the cognate virus and to closely related strains and viruses with high sequence homology (Baulcombe, 1996; Burgyán, 2006; Lindbo and Dougherty, 2005; McDiarmid, 2005; Voinnet 2001, 2005).

The constitutive expression of viral genes, however, does not occur in most conventional crops. Therefore, environmental safety issues have been raised on potential risks associated with the release of virus-resistant transgenic crops. Of major concern is the possibility of recombination between viral transgene transcripts and the genome from field viruses, which challenge transgenic plants (Aaziz and Tepfer, 1999; Allison *et al.*, 1996; de Zoeten, 1991; Falk and Bruening, 1994; Fuchs and Gonsalves, 1997, 2002; Hammond *et al.*, 1999; Hull, 1989; Martelli, 2001; Miller *et al.*, 1997; Rissler and Mellon, 1996; Robinson, 1996; Rubio *et al.*, 1999; Tepfer, 2002; Tepfer and Balazs, 1997). Resulting recombinant viruses may have identical biological properties as their parental lineages or new biological properties such as changes in vector specificity, expanded host range, and increased pathogenicity. The presence of constitutively expressed viral genes in transgenic plants has even been hypothesized to enhance the rate of plant virus evolution through recombination (de Zoeten, 1991; Hull, 1989; Jakab *et al.*, 1997; Rissler and Mellon, 1996). Recombination is a natural phenomenon that constitutes an important source of variation and a major driving force in the evolution of plant viruses (Chare and Holmes, 2006; Froissart *et al.*, 2005). Therefore, the issue of recombination did not arise with the development of transgenic plants expressing viral genes. Nevertheless, it is important to determine if recombination occurs in transgenic plants expressing viral genes beyond baseline events in conventional plants.

Recombination refers to the creation of chimeric RNA molecules from distinct segments present in different parental molecules, one donor and one acceptor (Hull, 2002). One mechanism underlying recombination is template switching during RNA replication in which the synthesis of a nascent RNA strand on a donor RNA molecule is halted. As a consequence, the RNA-dependent RNA polymerase or the nascent RNA strand can interact with the acceptor RNA molecule, leading to template switching and the creation of a chimeric RNA molecule. Another mechanism for recombination is template switching induced by pausing of the RNA-dependent RNA polymerase at break points on the RNA template. Recombination can be homologous when it occurs between two RNA molecules that are identical or very similar at the crossover point. It can also be heterologous when it occurs between two RNA molecules with limited or no obvious homology. Information on natural recombination rates in plant virus populations is becoming abundant. Recent studies have even indicated that the frequency of recombination can be very high for some viruses (Chare and Holmes, 2006; Froissart *et al.*, 2005; García-Arenal *et al.*, 2001). Also, in the case of *Potato virus Y* (PVY) in grapevines, recombination is thought to trigger the incorporation of viral sequences into the plant genome (Tanne and Sela, 2005).

Compelling evidence of recombination between viral transgene transcripts and viruses challenging transgenic plants has been obtained from laboratory and greenhouse experiments (Adair and Kearney, 2000; Borja *et al.*, 1999; Frischmuth and Stanley, 1998; Gal *et al.*, 1992; Greene and Allison, 1994, 1996; Jakab *et al.*, 1997; Lommel and Xiong, 1991; Schoelz and Wintermantel, 1993; Teycheney *et al.*, 2000; Varrelmann *et al.*, 2000; Wintermantel and Schoelz, 1996). Limited information, however, is available on the potential of transgenic plants to mediate the development of recombinant viruses under field conditions, although a few reports indicated no apparent effect of transgenic crops on the emergence of recombinant viruses (Fuchs *et al.*, 1998; Lin *et al.*, 2001; Thomas *et al.*, 1998).

To address the issue of recombination under field conditions, a network of scientists from various European countries and institutions, including France, Germany, Italy, Romania, Slovenia, and Spain, was formed. Their work was sponsored by the European Commission under the 5th framework competitive grant program. Transgenic plums and grapevines expressing viral CP genes were used as model systems. The objective of the project was to: (i) analyze and compare the dynamics and variability of virus populations in transgenic versus conventional plants under field conditions, (ii) monitor the emergence of recombinant virus species, (iii) examine whether transgenic grapevines and plums expressing viral CP genes increase the likelihood of emergence of recombinant viruses beyond that of natural background events. In addition, we also evaluated both the stability of post-transcriptional gene silencing (PTGS) in transgenic plums following multiple virus infection, and the translocation of transgene-derived products from transgenic grapevine rootstocks to nontransgenic scions.

These studies were carried out over 3.5 years (2003 to 2006) to provide realistic insights into the significance of recombination in transgenic plants expressing viral genes and the stability of engineered virus resistance under field conditions. We summarize here our major findings and discuss their relevance in regard to the safe use of transgenic crops expressing viral genes for disease management.

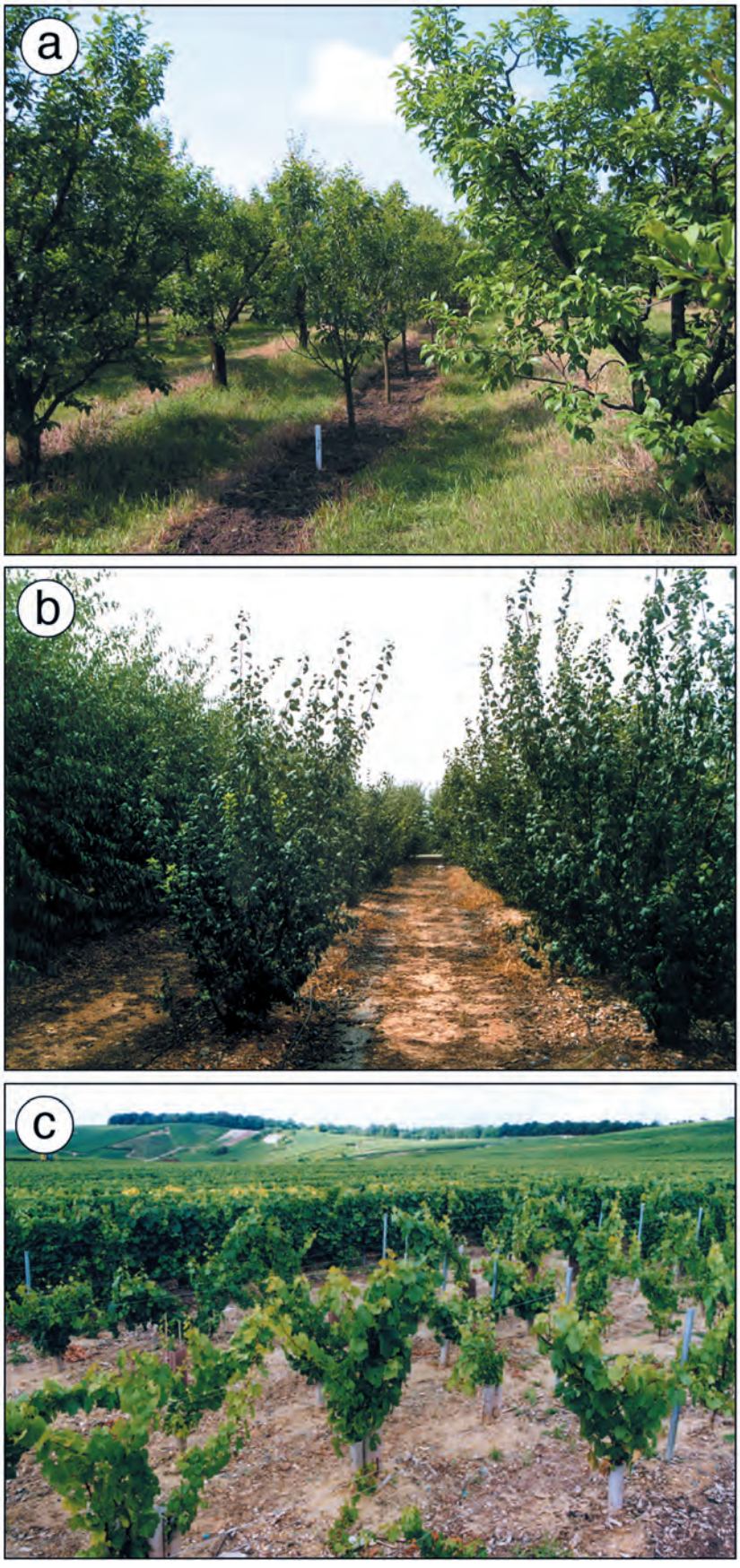

Fig. 1. (a) Transgenic plum trees expressing the coat protein gene of *Plum pox virus* in an experimental orchard in Romania and (b) Spain, and (c) transgenic grapevines expressing the coat protein gene of *Grapevine fanleaf virus* in an experimental vineyard in France.

TEST MATERIAL AND EXPERIMENTAL APPROACH

Test material consisted of transgenic plums expressing the CP gene of the potyvirus *Plum pox virus* (PPV) and transgenic grapevines expressing the CP gene of the nepovirus *Grapevine fanleaf virus* (GFLV), and the vitiviruses *Grapevine virus A* (GVA) or *Grapevine virus B* (GVB). Conventional plums and grapevines were used as control. Orchard and vineyard sites with transgenic plants were selected in France, Romania, and Spain. In addition, virus-infected conventional plums and grapevines were selected in orchards and vineyards in Germany, Italy, Slovenia, and other parts of Europe, and surveyed to determine a baseline level of the genetic variability of viral populations under field conditions.

The experimental approach was based on a comparative characterization of the biological, serological, and molecular properties of viruses infecting transgenic and conventional plants. The CP gene and eventually other genes of challenge virus isolates were amplified from test plants by immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) or RT-PCR using appropriate antibodies and primers, and further characterized by restriction fragment length polymorphism (RFLP) or single-stranded conformation polymorphism (SSCP), and nucleotide sequencing after cloning.

SAFETY ASSESSMENT OF TRANSGENIC PLUMS EXPRESSING THE PPV CP GENE

Transgenic plum trees expressing the PPV CP gene and conventional plums were tested in experimental orchards under conditions of high PPV inoculum pressure in Romania (Figure 1a) and Spain (Figure 1b). Transgenic C5 plum trees (Ravelonandro *et al.*, 1997), which display PTGS (Hily *et al.*, 2004; Scorza *et al.*, 2001), were highly resistant to graft-inoculation with PPV and no tree became infected via aphid-mediated transmission of PPV over an eight (1997-2006) or a 10-year field trial in Spain (Malinowski *et al.*, 2006) and Romania (1996-2006) (Zagrai *et al.*, 2007a, b), respectively. These data confirmed the durability of resistance to PPV of C5 plum trees in Poland (Malinowski *et al.*, 2006). Other transgenic plum lines tested were susceptible to PPV infection although transgenic C4 trees exhibited a significant delay in disease onset. No statistically significant difference in serological and molecular characteristics in the viral RNA 3' end region, i.e. the NIb and CP genes, was found among the majority of PPV isolates infecting transgenic and conventional plums in Romania (Zagrai *et al.*, 2007a, b) and Spain (Capote *et al.*, 2007). Similar data were obtained under greenhouse conditions (Kundu *et al.*, 2005). Also, no statistically significant difference was found in aphid species, number of aphids, including viruliferous individuals, and other arthropods visiting transgenic and conventional plums in Spain (Capote *et al.*, 2006).

Interestingly, several naturally occurring PPV recombinant isolates were found in conventional and PPV-susceptible but not in PPV-resistant transgenic plums in Romania (Zagrai *et al.*, 2007b). These PPV recombinants did not arise from recombination events with PPV CP gene transcripts in transgenic plums because they did not carry sequence motifs characteristic of the PPV CP transgene. Instead, they were highly similar to PPV-Rec isolates that emerge from recombination between PPV-D and PPV-M isolates (Glasa *et al.*, 2002, 2004). Therefore, transgenic plums did not alter the diversity and dynamics of PPV populations to detectable levels, nor did they trigger the emergence of recombinant PPV species. Furthermore, graft-inoculation of transgenic plums expressing the PPV CP gene with the ilarvirus *Prunus necrotic ringspot virus* (PNRSV) and PPV, or the trichovirus *Apple chlorotic leaf spot virus* (ACLSV) and PPV, or the ilarvirus *Prune dwarf virus* (PDV) and PPV in the field (Capote and Cambra, 2005; Zagrai *et al.*, 2007c) and in the greenhouse (Ravelonandro *et al.*, 2007; Zagrai *et al.*, 2007c) did not affect the efficacy and stability of PTGS over a three-year period. As a consequence, resistance to PPV did not break down.

SAFETY ASSESSMENT OF TRANSGENIC GRAPEVINES EXPRESSING VIRAL GENES

Transgenic and conventional grapevines were tested over a three-year period in two naturally GFLV-infected vineyard sites in France (Figure 1c). A few transgenic lines exhibited resistance to GFLV while most of them were as susceptible as conventional grapevines to *Xiphinema index*-mediated GFLV infection (Vigne *et al.*, 2004a). No characteristics similar to GFLV strain F13, which provided the CP transgene, were found in GFLV isolates challenging transgenic grapevines (Vigne *et al.*, 2004a). Furthermore, no statistically significant difference in molecular variability was detected for the majority of GFLV isolates from transgenic and conventional grapevines (Vigne *et al.*, 2004b). Therefore, transgenic grapevines did not assist the emergence of viable GFLV recombinants to detectable levels nor did they affect the molecular diversity of indigenous GFLV populations during the trial period (Vigne *et al.*, 2004a, b).

Analysis of GFLV isolates from different conventional cultivars and various geographic origins provided the baseline information of molecular variability (Pompe-Novak *et al.*, 2007; Vigne *et al.*, 2004a, b). Interestingly, GFLV recombinants were identified in conventional plants that were located either outside of the two field sites where transgenic plants were tested or in other vineyard sites in France (Vigne *et al.*, 2004a, b; 2005;

2006) and Slovenia (Pompe-Novak *et al.*, 2007). Recombination events were detected in the three genes encoded by GFLV RNA-2 with no hot spot of recombination (Pompe-Novak *et al.*, 2007; Vigne *et al.*, 2004a, b; 2005). Also, a recombinant isolate between GFLV and *Arabis mosaic virus* (ArMV), a closely related nepovirus, was detected in conventional grapevines (Vigne *et al.*, 2006). One of the GFLV recombinant isolates and the interspecies GFLV-ArMV recombinant isolate had similar biological properties to nonrecombinant GFLV isolates (Vigne *et al.*, 2005; 2006).

In addition, no clear association was found between symptomatology in *Vitis vinifera* cv. Volovnik and genetic variability, including the occurrence of recombination, within GFLV RNA-2 (Pompe-Novak *et al.*, 2007). Furthermore, no marked difference was found in the nucleotide composition of the CP gene of ampelovirus *Grapevine leafroll-associated virus 1* (GLRaV-1) isolated from either conventional or transgenic grapevines expressing the GFLV CP gene (W. Jelkmann and C. Mikona, unpublished information). Also, no compelling evidence was found for the translocation of GFLV-derived transgene products, i.e. protein, transcripts and small interfering RNAs (siRNAs), from transgenic grapevine rootstocks expressing the CP gene of GFLV to non-transgenic grapevine scions by DAS-ELISA, RT-PCR, and northern hybridization with enriched low molecular weight total RNA and a ^{32}P-labelled GFLV CP probe, respectively (Laval *et al.*, 2004). Finally, a RNA-silencing suppressor was identified in the genomic RNA of GVA (Zhou *et al.*, 2006) and transgenic grapevines expressing the CP gene of GVA or GVB that were challenge inoculated either with the homologous or the heterologous vitivirus by grafting or viruliferous mealybugs did not have any significant impact on the genetic variability of GVA and GVB populations (Sciancalepore *et al.*, 2007). Therefore, the original variability of challenge vitiviruses was preserved in transgenic grapevines even in the presence of high transgene transcript accumulation and systemic plant infection (Sciancalepore *et al.*, 2007).

REAL ENVIRONMENTAL IMPACT OF TRANSGENIC PLUMS AND GRAPEVINES ENGINEERED FOR VIRUS RESISTANCE

Our field studies provided strong evidence that transgenic plums and grapevines are not only highly resistant to PPV and GFLV over 10 and 3 years, respectively (Capote *et al.*, 2006; Malinowski *et al.*, 2006; Vigne *et al.*, 2004a; Zagrai *et al.*, 2007b), but also do not have adverse effect on the diversity and dynamics of virus populations via recombination beyond natural background events (Capote *et al.*, 2006; Kundu *et al.*, 2005; Ravelonandro *et al.*, 2007; Vigne *et al.*, 2004a, b; Zagrai *et al.*, 2007a, b).

Providing a direct evaluation of the environmental impact of transgenic grapevines and plums expressing viral CP genes is the most valuable approach to safety assessment. Interestingly, our results indicate that field studies do not reflect most laboratory studies in regard to the occurrence of recombination in transgenic plants expressing viral genes. The stringency of selective pressure is likely accounting for differences observed between field and laboratory studies. Under laboratory conditions, high selective pressure is commonly used (Adair and Kearney, 2000; Barajas *et al.*, 2006; Borja *et al.*, 1999; Frischmuth and Stanley, 1998; Gal *et al.*, 1992; Greene and Allison, 1994; Lommel and Xiong, 1991; Schoelz and Wintermantel, 1993; Varrelmann *et al.*, 2000). These conditions are designed to enhance the likelihood of creating a detectable recombination event that is usually rare and, hence, not easy to identify. Conditions of high selective pressure are conducive to recover recombinant RNA and/or recombinant viruses at high rates, for instance, by assessing the ability of a defective challenge virus to spread systemically in transgenic plants expressing a complementary functional gene. In contrast, under conditions of moderate selective pressure, recombination rates are low (Allison et *al.*, 1996; Barajas *et al.*, 2006; Wintermantel and Schoelz, 1996) or even undetectable under conditions of low, if any, selective pressure (Adair and Kearney, 2000; Allison *et al.*, 1996; Capote *et al.*, 2006; Fuchs *et al.*, 1998; Lin *et al.*, 2001; Thomas *et al.*, 1998; Vigne *et al.*, 2004a, b; Zagrai *et al.*, 2007a, b). The effect of the strength of selective pressure applied to the challenge virus in favor of the recombinants has been recently confirmed in *Nicotiana benthamiana* displaying RNA silencing (Barajas *et al.*, 2006).

Our studies did not disclose the appearance of recombinant viruses to a detectable level in transgenic grapevines and plums that were grown in distinct open (France, Romania, and Spain) and confined (France, Germany, and Italy) environments, although our test plants were exposed to various vectors (aphids, mealybugs, and nematodes) and different viral populations (GFLV, GLRaV-1, GVA, and GVB in the case of grapevines, and ACLSV, PDV, PNRSV, and PPV in the case of plums) (Capote *et al.*, 2006; Kundu *et al.*, 2005; Ravelonandro *et al.*, 2007; Sciancalepore *et al.*, 2007; Vigne *et al.*, 2004a, b; Zagrai *et al.*, 2007a, b).

To the best of our knowledge, this is the first extensive risk assessment study of transgenic woody plants expressing viral genes under realistic conditions in multiple locations and environments. Our findings with perennial crops provide important new insights into the environmental impact of transgenic plants expressing viral CP genes, and expand on earlier field reports with vegetable crops (Fuchs *et al.*, 1998, 1999; Lin *et al.*, 2001; Thomas *et al.*, 1998). Based on the currently available information from field experiments conducted un-

der natural conditions of low selective and high disease pressure, it seems clear that the likelihood of recombinant viruses to emerge in transgenic plants expressing viral genes is extremely low, even not measurable, regardless of the crop, viral transgene construct, challenge virus, vector species, mode of transmission, and environmental conditions (Fuchs *et al.*, 1998, 1999; Thomas *et al.*, 1998; Lin *et al.*, 2001; Vigne *et al.*, 2004a, b; Capote and Cambra, 2005; Capote *et al.*, 2006; Zagrai *et al.*, 2007a, b). Therefore, recombination with viral transgene transcripts seems to be less significant than initially predicted (Hull, 1989; de Zoeten, 1991; Rissler and Mellon, 1996; Jakab *et al.*, 1997).

CONCLUSIONS

Our studies conducted under open and confined environments did not indicate that transgenic grapevines and plums assist the emergence of recombinants or increase the frequency of recombinant viruses and the creation of more severe virus variants, in particular in comparison to mixed infected conventional plants. Thus, there is little, if any, evidence to suggest that transgenic plants expressing viral genes favor the emergence of new viruses that could not arise naturally from mixed infected conventional plants (Falk and Bruening, 1994; Fuchs and Gonsalves, 1997, 2002; Hammond *et al.*, 1999; Martelli, 2001; Miller *et al.*, 1997; Rubio *et al.*, 1999). Also, PPV-resistant transgenic plums did not affect the dynamics and diversity of aphid and other arthropod populations (Capote *et al.*, 2006). This neutral impact on non-target pests was expected as plant viral CPs are not known to influence directly insect populations. Altogether, our studies (Capote and Cambra, 2005; Capote *et al.*, 2007; Kundu *et al.*, 2005; Laval *et al.*, 2004; Pompe-Novak *et al.*, 2007; Ravelonandro *et al.*, 2007; Sciancalepore *et al.*, 2007; Vigne *et al.*, 2004a, b; 2005; Zagrai *et al.*, 2007a, b, c) confirm earlier predictions on limited, if any hazard, associated to transgenic plants expressing viral genes (Allison *et al.*, 1996; Borja *et al.*, 1999; Falk and Bruening, 1994; Fuchs and Gonsalves, 2002; Hammond *et al.*, 1999; Martelli, 2001, Miller *et al.*, 1997; Robinson, 1996).

We expect our work to further advance our understanding on the safety of virus-resistant transgenic plants. It is important to keep in perspective that resistance to devastating viruses is of great interest to the grapevine and stone fruit industries, and to agriculture in general in Europe and worldwide. Given the pressing need for effective and environmentally-friendly virus control strategies, we expect our findings to assist national and international authorities in making scientifically-based regulatory decisions for the timely release of virus-resistant grapevines and plums, and of other transgenic crops that contain viral genes and are of agronomic importance. PPV-resistant transgenic C5 plum, designated HoneySweet, is currently being considered for deregulation in the United States of America (Scorza *et al.*, 2007). Our findings are timely to help balance risks and benefits of this effective approach to virus control.

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