Silencing in Genetically Engineered *Prunus domestica* Provides Durable and Safe Resistance to *Plum pox virus* (Sharka Disease)

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**Abstract**

Originally identified in Bulgaria in 1915, *Plum pox virus* (PPV) is the most damaging virus of stone fruit trees, including apricot, plum, peach and cherry. PPV steadily spread throughout Europe over the years since its discovery and at the turn of the century (1999-2000) it reached North America (USA and Canada). While many strategies to control the spread of PPV have been undertaken over the decades and many studies have contributed to the characterization of the virus isolates there has been relatively little progress in the development of resistant varieties. With the paucity of natural resistance, transgenic technology, based on the engineering of the virus capsid gene, was investigated as a useful source of resistance. This work identified the C5 plum clone as highly resistant to PPV infection. These findings were supported by detailed molecular studies indicating that post-transcriptional gene silencing (PTGS) is the resistance mechanism with resistance being mediated through the production of small interfering RNA (siRNA). The durability of PPV resistance in C5 (named ‘HoneySweet’) is reflected through more than 10 years of field tests. In total, over 15 years of research with ‘HoneySweet’ have demonstrated that this clone and the resistance mechanism that it represents is: i) an important tool to demonstrate the successful deployment of biotechnology against a quarantine pest; ii) a safe product of biotechnology; and iii) a useful strategy for avoiding the use of pesticides to control natural aphid vectors of PPV. The deregulation of ‘HoneySweet’ in the USA by USDA/APHIS (Federal Register Doc. E7-13649, July 12 2007) and clearance by the U.S. Food and Drug Administration (FDA) corroborate these findings.

**INTRODUCTION**

*Plum pox potyvirus* (PPV) causes severe disease impacts in *Prunus* (Cambra et al., 2006). Presently, fruit tree growers do not have reliable control measures. Studies of the virus including strain variability, and new serological tools and updated molecular techniques (Real-time, QPCR) (Schneider et al., 2004) have been important in terms of our knowledge of this disease, yet they have had little effect on the spread of the disease throughout Europe and into areas such as North and South America, Asia, and North Africa (EPPO/OEPP Bulletin, 2006).

There is neither cure nor treatment against PPV and few *Prunus* cultivars have been characterized as highly resistant. While the resistant clones that have been identified are being incorporated in breeding programs (Hartmann and Neumüller, 2006) these are long-term efforts and do not solve the present problems associated with PPV spread. This suggests the utility of biotechnology as an alternative or complementary breeding approach to achieving high level resistance to PPV in *Prunus*. This approach has been well-demonstrated in solving the papaya ringspot virus problem in Hawaii (Gonsalves, 2006).

Scorza et al. (1994) engineered the PPV CP gene via *A. tumefaciens* into plum (*P. domestica*). They discovered that resistance was the result of post-transcriptional gene silencing (PTGS) (Scorza et al., 2001; Hily et al., 2004) mediated by siRNA produced by...
a hairpin arrangement of the inserted CP transgene (Hily et al., 2005; Kundu et al., 2008). The resistance of clone C5, later named ‘HoneySweet’ (Scorza et al., 2007) was shown to be of a high level and durable for over 10 years in field tests in Europe (Ravelonandro et al., 2002; Malinowski et al., 2006; Polak et al., 2008; Zagrai et al., 2008). Risk assessment studies of ‘HoneySweet’ and other PPV-CP transgenic lines, showed that virus and viral transgene interactions are not a source of new emerging viruses (Capote and Cambra, 2005; Capote et al., 2008; Ravelonandro, 2006; Fuchs et al., 2007) and that the transgenic trees do not affect the population structures of aphid vectors (Capote et al., 2008).

This report reviews how ‘HoneySweet’ plum transformed with the PPV CP transgene is durably resistant to PPV infection. Recognized as the key-factor of PPV resistance, we show how silencing is not only detected in vegetative tissues but also in plum fruits. The last section of the report discusses the safe use of transgenic fruit-trees indicating that such trees can be considered as an important strategy to control quarantine pests such as PPV.

MATERIALS AND METHODS

Plant Material and Virus Inoculation

Transgenic plum clones C-2, C-3, C-4, C-5 and C-6 containing the PPV CP gene (Scorza et al., 1994) were vegetatively propagated onto rootstocks. Virus resistance experiments were initially conducted in greenhouses. Following four artificially-induced dormancy cycles, resistant plums were identified (Ravelonandro et al., 1997). To validate these observations, the same clones were transferred to the field along with relevant non-transgenic controls. Tests were conducted in different European countries including sites in continental (Poland, Romania, Czech Republic) and Mediterranean (Spain) areas (Ravelonandro et al., 2002; Malinowski et al., 2006; Capote et al., 2008; Zagrai et al., 2008). The rationale was to challenge plants in different ecological conditions and with native PPV strains. These factors have been indicated as important parameters for assessing PPV resistance (Kegler et al., 1998). These plums were then assayed either by graft- or aphid-inoculation (Ravelonandro et al., 2002; Malinowski et al., 2006; Capote et al., 2008; Zagrai et al., 2008).

Biosafety Studies

When transgenic plants expressing viral genes began to be extensively developed, potential biological risks were proposed based on the use of CP genes (Hull, 1990). These potential risks were heteroencapsidation (Lecoq et al., 1993; Fuchs and Gonsalves, 2007) and recombination (Aaziz and Tepfer, 1999). ‘HoneySweet’ plum transgene silencing was shown to eliminate the production of CP and therefore the potential for heteroencapsidation in vegetative tissues (Hily et al., 2005). Silencing in fruit tissue was not reported and remained as an area of investigation along with the potential for recombination (in fruit and vegetative tissues) which could result from transgene mRNA production alone.

RESULTS AND DISCUSSION

siRNA-Induced PTGS Confers the High Level and Durable Nature of Resistance in ‘HoneySweet’ Plum

Some of the transgenic clones developed by Scorza et al. (1994) expressed CP (C-2,-3 and -4) and two clones, C-5 (‘HoneySweet’) and C-6 do not. Initial greenhouse results revealed the highly resistant behaviour of clone C-5 (Ravelonandro et al., 1997). It was also observed that plum clones expressing the virus CP gene were susceptible. Scorza et al. (2001) expanded the analysis of the molecular mechanisms involved and found that the resistance to PPV infection was associated with PTGS, a natural regulation system that was initially reported with the silencing of the chalcone synthase gene in petunia.
Comparative studies with samples collected both from greenhouse and field indicated that these molecular phenomena are associated with the hypermethylation of the viral transgene (Hily et al., 2004). Following the progress achieved in virus transgene studies in herbaceous plant models, Hily et al. (2005) demonstrated that PTGS displayed by plum C-5 followed the process reported in herbaceous plant models (Hamilton et al., 2002). C-5 plum produced double stranded RNA duplexes of 21 and 26nt (Hily et al., 2005). Scorza et al. (2007) have reported in the USDA APHIS deregulation dossier that in C-5 two viral transgene copies are naturally rearranged in tandem in a manner that would lead to a double stranded intron hairpin (ihp) mRNA structure that when cleaved would produce siRNAs. In the cytoplasm, siRNA interacts with the RNA-induced silencing complex (RISC) that cleaves the PPV RNA genome into oligonucleotides and prevents virus infection. Kundu et al. (2008) have shown that the 26-nt dsRNA is responsible for systemic silencing and this explains why all C-5 plums exposed to natural infection in the field are resistant.

Studies utilizing new hairpin constructs (Hily et al., 2007) have confirmed the activity of these structures in inducing PPV resistance in plum.

**Biosafety of Transgenic Plum-Trees**

Considering that the transgenic plum trees were in field tests for at least 10 years these plantings provided an excellent experimental system for the study of the virus populations in a woody perennial crop harbouring a virus CP gene (Fuchs et al., 2007). Through intensive leaf sampling of those transgenic plums expressing transgene CP, studies focused on the nucleotide sequences spanning the nuclear inclusion “b” (Nib) to CP cistrons of the challenge PPV strain. Strikingly, a very low percentage of nucleotide substitution occurred in the PPV challenge strain(s) sampled either in greenhouse (Ravelonandro, 2006) or field tests (Capote and Cambra, 2005; Zagrai et al., 2008). Moreover, aphid vectors of PPV did not distinguish between transgenic and conventional plum trees (Capote et al., 2008). In both Spain where only the D strain was found and in Romania where D, M, D+M, and Rec were identified the results showed that there were no significant changes in the virus nucleotide sequences in transgenic as compared to conventional plums (Capote et al., 2008; Zagrai et al., 2008). These studies indicated that there are no unexpected effects of transgene CP production on recombination and confirm the bio or environmental-safety of ‘HoneySweet’.

**siRNA Detected in HoneySweet Plum Fruits**

When the expression of marker genes associated with PPV CP (Scorza et al., 1994) was analysed in fruits, as expected, both NPTII and GUS were detected by immunoblotting. Figure 1 shows that fruits accumulate less GUS protein when compared to detection in leaf tissue (NPTII not shown).

No CP was detected in ‘HoneySweet’ fruits (not shown) but it was not known if the siRNA complex was present in fruit. To study siRNA presence in fruit they were separated into young green and mature stages of development. While the siRNA duplex was detected in young green fruits, we failed to detect these molecules in mature fruits (Fig. 2). Attempts to detect siRNA in mature fruits are still being pursued in order to determine if siRNA may be produced in specific tissues such as the epidermis or the mesocarp.

**CONCLUSIONS**

‘HoneySweet’ plum has been deregulated in the U.S. by the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS) and cleared by FDA. Approval by the U.S. Environmental Protection Agency (EPA) is pending. Before European growers can profit from the use of ‘HoneySweet’ it must be approved in the EU. In the EU some progress has been made in legislation that will provide for the eventual release of the transgenic crops, notably the co-existence of genetically modified crops with conventional and organic farming (EU recommendation...
2003/556/EC). But more can be done. If the goal is to create an artificial fence that would prevent the transfer of the virus transgene into conventional or wild species, such a consideration excludes the potential positive effects of the viral transgene. Clearly, viral transgene flow into related species can be perceived as advantageous rather than harmful (Fuchs and Gonsalves, 2007) In fact such a transfer in the case of ‘HoneySweet’ which is known to transfer resistance in a Mendelian fashion (Scorza et al., 1998) could provide protection to wild plum germplasm that is disappearing, in part, due to the effects of PPV infection and programs that eradicate infected trees (Botu et al., 2007).

Over 15 years of research with ‘HoneySweet’ plum has demonstrated the efficiency and safety of using genetic engineering technology to combat PPV. Its practical application to aid growers and to help guarantee the future availability of affordable plum production is the next important stage of activity.

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Literature Cited


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Fig. 1. Western-blotting analysis of plum C-4 and C-5 fruits for the detection of GUS (indicated by arrows). Lane 1 represents total protein extracts from leaves, lanes 2 and 4 came from fruit mesocarp, lanes 3 and 5 derived from small green fruits and lane 6 represents extract from mature fruit.

Fig. 2. Autoradiogram representing siRNA detection in ‘HoneySweet’ plum fruits: lane 1, mature; lane 2, small green and lane 3, leaves. 20 µg of total RNA extracted from fruits and leaves were loaded onto a denaturing polyacryamide gel. Following to the electro-transfert of RNA onto hybond-N+ membrane, 32P labelled CP RNA probe was used to detect siRNA through an overnight hybridization at 42°C. siRNA indicated by the two arrowheads, at the right margin, were then detected through autoradiography.