Plant Cell Tiss Organ Cult
DOI 10.1007/s11240-013-0339-6

REVIEW

Genetic engineering of *Plum pox virus* resistance: 'HoneySweet' plum—from concept to product

Ralph Scorza · Ann Callahan · Chris Dardick · Michel Ravelonandro · Jaroslav Polak · Tadeusz Malinowski · Ioan Zagrai · Mariano Cambra · Ivanka Kamenova

Received: 25 March 2013 / Accepted: 29 May 2013
© Springer Science+Business Media Dordrecht (outside the USA) 2013

Abstract Sharka disease, caused by *Plum pox virus* (PPV) was first recorded in Bulgaria during the early twentieth century and since that first report, the disease has progressively spread throughout Europe and more recently to Asia, Africa, North and South America. Few PPV resistance genes have been found to naturally occur in *Prunus* and this has led to the investigation of biotech approaches to the development of resistance through genetic engineering (GE). A notable example of the utility of this approach is 'HoneySweet' plum. PPV protection in this case is based on RNA interference (RNAi) and resistance has been shown to be highly effective, stable, durable, and heritable as a dominant trait. Extensive testing and risk assessment of 'HoneySweet' in laboratory, greenhouse and in the field for over 20 years has demonstrated not only the effectiveness but also the safety of the technology. 'HoneySweet' has been cleared for cultivation in the USA. By the appropriate regulatory agencies. The development and regulatory approval of 'HoneySweet' demonstrate the ability of RNAi technology to contribute to the sustainability of stone fruit production in regions impacted by PPV. Although it has taken almost 100 years since the identification of sharka, we are now able to effectively protect stone fruit species against this disease through the application of GE.

Keywords Fruit breeding · Genetic engineering regulation · Genetic Modification · Pathogen-derived resistance · Plum · Prunus domestica · Rapid cycle breeding · Risk assessment · RNAi · siRNA · Rosaceae · Sharka · Transgenic plants

R. Scorza (✉) · A. Callahan · C. Dardick
Agricultural Research Service-Appalachian Fruit Research Station, United States Department of Agriculture, Kearneysville, WV, USA
e-mail: Ralph.Scorza@ARS.USDA.GOV

M. Ravelonandro
UMR 1332 Biologie du Fruit et Pathologie, INRA-bordeaux, Villenave D'Ornon Cedex, France

J. Polak
Crop Research Institute, Prague, Czech Republic

T. Malinowski
Institute of Horticulture, Skierniewice, Poland

I. Zagrai
Fruit Research and Development Station Bistrita, Bistrita, Romania

M. Cambra
Instituto Valenciano de Investigaciones Agraria (IVIA), Moncada, Valencia, Spain

I. Kamenova
AgroBioInstitute, Sofia, Bulgaria

Introduction

Plum pox virus (PPV) is the most serious viral disease of the genus Prunus. Because of this, there is a vast amount of literature concerning PPV covering virtually all aspects of this organism's biology and its interactions with its hosts. This review focuses on the development of host plant resistance to this virus through genetic engineering, and specifically, resistance based on RNA interference (RNAi). In the past decade the literature on RNAi has expanded exponentially. The application of this technology to PPV resistance has been recently and comprehensively reviewed (Ilardi and Di Nicola-Negri 2011). In the context of the biology of PPV and RNAi technology, the current review

Published online: 12 June 2013

seeks to present an example of the use of RNAi to provide long-term, stable protection against PPV in a commercial *Prunus* species (*P. domestica*) by reviewing the process from the development of the concept, through the research and development pipeline, through the US regulatory process, and exiting the pipeline with a commercially viable PPV resistant plum variety.

Plum pox virus

Sharka disease caused by PPV was first observed in Bulgaria during the First World War in 1917 and described by Atanassoff (1932). By the late 1970s the disease had spread throughout Europe (Roy and Smith 1994) and despite the considerable efforts made in respect to sharka containment, the disease has been reported in stone fruit producing countries worldwide except Australia, New Zealand and South Africa (Table 1) (Barba et al. 2011; OEPP/EPPO Bull 2006).

Plum pox virus (PPV) is classified in the family *Potyviridae* and the genus *Potyvirus* and has been well characterized molecularly (James and Glasa 2006; Olmos et al. 2006). PPV hosts include cultivated, ornamental and wild *Prunus* species (Damsteegt et al. 2007). Non-*Prunus* species, in at least nine plant families, have been infected artificially with one or more strains of the virus, and in some cases have been found naturally infected in the field (van Oosten 1971; Llácer 2006). Generally, PPV affects the fruits of sensitive cultivars of the commercially cultivated stone fruits such as plums (*P. domestica, P. salicina*), apricots (*P. armeniaca*), peaches and nectarines (*P. persica*). Cherries (*P. avium and P. cerasus*) can be infected by PPV C strain (Crescenzi et al. 1995; Kalayshan et al. 1994; Nemchinov and Hadidi 1996) and almond (*P. dulcis*) can also be infected (Llácer and Cambra 2006). Symptoms of sharka disease vary widely depending on the crop, the cultivar, the virus strain, and the environment. Affected plum fruits are deformed and show deeply engraved rings, irregular lines, and fruit malformation. The flesh turns brownish red and is saturated by gum and is unsuitable for marketing (Fig. 1). Up to 90 % of fruit fall prematurely and these fruit are unsuitable both for direct consumption and for industrial processing (Kegler and Schade 1971; Kegler and Hartmann 1998). Similar symptoms of PPV infection are common on apricot, peach, nectarine, and other stone fruits. Most stone fruit varieties show leaf symptoms which appear as pale or yellow green rings, diffuse spots or leaf mottling, chlorotic oak-leaf patterns, shallow depressions, and shoots may split and die back. Peach and plum blossoms of PPV-infected trees can also show symptoms, consisting of darker, pink colored speckles on the pink colored petals of the peach (http://web.pppmb.cals.cornell.edu/fuchs/ppv/ppv_symptoms.php). Highly sensitive plum cultivars such as 'Ortenauer', in addition to fruit and leaf symptoms, show bark splitting and cankers on the shoots that become dry and brittle and die. Infected highly susceptible trees decline within only a few years (Kegler and Hartmann 1998).

The main pathway of long-distance PPV spread is through the use of infected propagative material and the distribution of infected trees. Once sharka has become established in an orchard aphids transmit the virus locally.

Table 1 World-wide spread of PPV as recorded in the literature

Country or region	Year of detection	Citation
Bulgaria	1917	Atanassoff (1932)
Bohemia (former Czechoslovakia)	ND	Blattný (1930)
Moravia (former Czechoslovakia)	ND	Atanassoff (1932)
Former Yugoslavia	1935	Josifović (1937)
Hungary	1938	Szirmai (1948)
Romania	1922	Minoiu (1997)
Albania	1947	Papingyi (1965)
Germany	1956	Schuch (1957)
Austria	1961	Vukovits (1961)
Poland	1958	Szczygieł (1962); Pieniazek (1962)
Moldova	1962	Verderevskaya (1965)
Netherlands	1966	Meijneke (1967)
Greece	1967	Demetriades and Castimbas (1968)
United Kingdom	1968	Cropley (1968)
Turkey	1968	Sahtiyanci (1969)
France	1970	Desvignes and Morvan, personal communication
Sweden	ND	Anonymus (1970)
Italy	1973	Canova et al. (1977)
Belgium	1974	Maroquin and Rassel (1976)
Spain	1984	Llácer et al. (1985)
Portugal	1984	Louro and Corvo (1985)
Egypt	1984	Dunéz (1987)
Syria	1984	Dunéz (1987)
Cyprus	1984	Dunéz (1987)
Chile	ND	Acuna (1993)
Azores	ND	Mendonca et al. (1997)
India	ND	Thakur et al. (1994)
USA	1999	Levy et al. (2000)
Canada	2000	Thompson et al. (2001)
Argentina	2004	Dal Zotto et al. (2006)
Kazakhstan	2003	Spiegel et al. (2004)
China	ND	Navratil et al. (2005)
Pakistan	ND	Kollerova et al. (2006)
Japan	2010	Maejima et al. (2010)

ND: year of detection not determined

Fig. 1 *Plumpox virus* symptoms: (*left*) fruit marking and deformation on mature *P. domestica* fruit (*right*) typical chlorotic ring development on *P. domestica* leaves

More than twenty aphid species are known to transmit PPV (Labonne et al. 1995; Gildow et al. 2004; Isac et al. 1998; Wallis et al. 2005). PPV is a non-persistently transmittable virus (Kunze and Krczal 1971). Aphids can acquire the virus from infected material in as short as 30 s and can transmit it for up to 2–3 h after acquisition (Moreno et al. 2009). These short periods are sufficient for spreading the virus within nurseries or orchards. PPV can be spread in orchards by transient aphids as efficiently as aphids colonizing *Prunus* species (Labonne et al. 1995). The speed of natural spread in orchards depends on the distance of the healthy trees to the infection sources. It has been shown that within a 100 m diameter from an infected individual tree, 48–100 % of the previously healthy trees have become infected within 10 years (Jordovic 1968). Systemic spread of the virus within a tree may take several years (OEPP/EPPO 1983). The virus may be detected in seed coats and cotyledons, but embryonic tissue and seedlings obtained from germinated seeds have not shown infection, therefore, PPV is not considered to be seed transmitted (Pasquini et al. 1998, 2000; Glasa et al. 1999; Polák and Komínek 2001; Pasquini and Barba 2006; Milusheva et al. 2008; Zagrai and Zagrai 2008).

The reduced fruit quality and premature fruit drop caused by sharka disease make stone fruit production in some European countries problematic (Kamenova et al. 2010). Cambra et al. (2006) have summarized production and losses in countries affected by PPV. Production for European plums was 3 million metric tons (mt) and losses were 1.5 million mt per year with an approximate value of €5,400 M over a 30 year period. The loss of apricot fruit was 0.6 million mt per year with an approximate value of €3,600 M for a 30 year period. Sharka has changed the landscape of stone fruit cultivars. For example, as early apricot cultivars are much more sensitive in terms of symptom expression on fruits than the late cultivars, many early local cultivars have progressively been substituted by later ripening tolerant varieties that show few or no symptoms on fruit (Cambra et al. 2006).

In countries not affected by PPV or with localized and quarantined infection zones primary focus is placed on preventing the introduction of PPV into uninfected fruit-growing areas. The development and enlargement of the European Union (EU) with increased movement and trade between countries with different levels of PPV infection and with different strains of the virus is likely responsible for the spread of PPV in general, and the wider distribution of the various PPV strains. The movement of PPV infected plant material from infected to non-infected or only partially-infected countries in Europe, along with the potential for spread of the more severe strains of PPV have been causes of great concern. Over the past 25 years over €33 M has been spent in Europe on programs to conduct research on sharka control (Cambra et al. 2006) including the recent SHARCO project funded at over €3.5 M that specifically addressed "Sharka containment in view of EU-expansion" (http://www.sharco.eu/sharco/ accessed March 19, 2013).

To prevent the long-distance spread of PPV within a state, country, or region, quarantine has been the first line of defense. Another element of the strategy in preventing virus introduction to a new area is the use of certified planting stock that has been tested and verified to be free of PPV (Polák et al. 1992). Unfortunately, visual inspections carried out on PPV tolerant cultivars which remain symptomless while infected have little value in terms of quarantine or certification programs. Once PPV is introduced into a new area the next control measure is the elimination of virus infected plants as quickly as possible before the disease spreads. Since the infected trees cannot be cured or the virus eliminated, infected trees must be eradicated.

PPV in North America

In December 1999, PPV was detected in peach and plum trees in orchards in Adams County, Pennsylvania (Levy et al. 2000). This detection resulted in what was to become a 10-year eradication program that cost over $65 M and resulted in almost the complete elimination of stone fruits in the affected Pennsylvania counties. In 2000 PPV was detected in Canada and after a decade long unsuccessful eradication effort PPV is now under a 5 year monitoring

and management program (http://www.inspection.gc.ca/plants/plant-protection/diseases/plum-pox-virus/monitoring-and-management-program/eng/1323887724804/1323889930176, accessed March 21, 2013). While only one strain was detected in Pennsylvania (PPV-D), three strains of PPV have been detected in Canada; PPV-D, Rec, and W (Thompson et al. 2001, 2009; James et al. 2003; James and Varga 2005, 2011). The future status of PPV in Canada is uncertain. Although PPV was eradicated in Pennsylvania it has since appeared in New York State in 2006 close to the border with Canada (http://www.agriculture.ny.gov/PI/ppv/ppv.html accessed March 18, 2013). The potential spread of PPV in the US and the presence of PPV on the northern border is cause for great concern for stone fruit production in the US *P. domestica* plums are particularly susceptible. California produces nearly 100 % of US dried plums and accounts for roughly 60% of world dried plum production, exporting to approximately 70 countries (http://www.agmrc.org/commodities__products/fruits/prunes-profile/, accessed March 19, 2013). In 2012 there were 55,000 bearing acres of *P. domestica* in the California producing 120,000 tons with a value of $164.4 M (http://www.nass.usda.gov/Statistics_by_State/California/Publications/Fruits_and_Nuts/201206prunf.pdf, Accessed March 19, 2013). PPV presents a serious risk to this industry. The need for PPV host-plant resistance is clear.

Resistance breeding

If eradication of PPV from an area is not possible and trees cannot be adequately protected from virus transmission by aphids due to the non-persistent mode of transmission, host plant resistance to the virus is the only remaining viable control strategy and is, in fact, the most sought-after means of control. Despite the fact that resistance to PPV has been pursued ever since the disease was first identified, there are few reports of high level resistance in commercial *Prunus* species. While resistance to PPV in genotypes of European plum have been reported these have generally not been durable or effective against all virus strains (Kegler et al. 1985, 1995). No immune or highly resistant genotypes within *P. domestica*, *P. spinosa* and *P. insitita* have yet been found. While most *P. cerasifera* germplasm is PPV susceptible one resistant clone has been identified but the basis of resistance or its durability is unknown (Minoiu et al. 1998). The lack of high level resistance in cultivated *P. domestica* plums has directed the attention of breeders towards hypersensitivity as a means of resistance. The cultivar 'Jojo' (Hartmann and Petruschke 2000; Hartmann and Neumüller 2006; Neumüller et al. 2010) which reacts to PPV inoculation with a hypersensitive reaction was introduced as the first completely resistant plum variety. While this variety appears to have exhibited a high level of resistance to PPV in the field there remain questions concerning the performance of trees under high inoculation pressures and the potential for virus movement through hypersensitive plants. Resistance may be virus strain-dependent and the virus can apparently move through the vessels of 'Jojo' (Polák et al. 2005a, b; Polák and Jarošová 2012). Observations in Romania have revealed that when young trees of 'Jojo' are fed upon by PPV-carrying aphids, tree death may occur as a result of the hypersensitive response (Zagrai, personal communication). The apparent multigenic nature of the hypersensitive response and the varying levels of hypersensitivity that occur in seedlings (Neumüller and Hartmann 2008; Lichtenegger et al. 2010) make breeding for this type of resistance a long-term process. Nevertheless, hypersensitivity represents a potentially valuable resistance mechanism that requires continued attention.

Naturally-occurring high level resistance to PPV has been identified in apricot originating from a small group of cultivars developed in North America (Polák and Komínek 2012) and these have been used as donors in the development of new PPV resistant apricot varieties through conventional breeding (Badenes and Llácer 2006; Dosba et al. 1992; Karayiannis et al. 1999). While the mechanism of resistance is as yet unknown, in order to improve selection efficiency, resistance has been mapped and molecular markers for PPV resistance in apricot have been developed (Salava et al. 2001, 2002; Dondini et al. 2011; Lalli et al. 2008; Lambert et al. 2007; Marandel et al. 2009; Soriano et al. 2012; Zhebentyayeva et al. 2008). High resolution mapping using simple sequence repeat markers has produced markers tightly linked to PPV resistance and use of these markers will increase the efficiency of the selection of PPV resistant apricot progenies (Soriano et al. 2012).

The paucity of high-level resistance to PPV in most *Prunus* species, incompatibility barriers between species, and long generation times associated with tree fruit breeding suggest that additional genetic improvement technologies are needed to develop stone fruit varieties highly resistant to PPV. Genetic engineering of papaya with the *Papaya ringspot virus* (PRSV) coat protein (CP) gene provided high levels of resistance to PRSV (another potyvirus) in a rapid and efficient manner and essentially saved the Hawaiian papaya industry (Gonsalves 1998; Ferreira et al. 2002). This strategy is generally referred to as pathogen-derived resistance (Sanford and Johnston 1985). Resistance may be the result of protein expression (Beachy et al. 1990) or RNA, specifically small interfering RNAs (Fire et al. 1998; Hamilton and Baulcombe 1999).

Prior to the discovery of PPV in the US, the United States Department of Agriculture-Agricultural Research

Service (USDA-ARS) began a program of pre-emptive breeding for PPV resistance. In 1989 researchers at the USDA-ARS Appalachian Fruit Research Station (AFRS), Kearneysville, West Virginia began work on the development of resistance to PPV through genetic engineering. Plums were first engineered with the *Papaya ringspot virus* (PRSV) coat protein (CP) gene (kindly provided by Dr. Dennis Gonsalves, Cornell University, Geneva, NY; currently USDA-ARS, Hilo, HI) which was used to develop PRSV resistant papayas (Gonsalves 1998; Ferreira et al. 2002). The PRSV-CP gene was thought to have sufficient homology to the PPV-CP gene to provide resistance to PPV. At the time that this work began, virus resistance was expected to be protein-mediated (Beachy et al. 1990). Protection against PPV in transgenic plums expressing PRSV-CP was effective for several years in greenhouse tests, but after 32 months trees became fully infected (Scorza et al. 1995). The PPV-CP gene was sequenced and cloned (Ravelonandro et al. 1992) and through a collaboration between D. Gonsalves, M. Ravelonandro and R. Scorza the PPV-CP gene was engineered into the plasmid pGA482GG (Fitch et al. 1990; Ling et al. 1991) previously used to engineer PRSV resistant papayas. In the US transgenic plants were inoculated with PPV in a containment greenhouse under a USDA-Animal and Plant Health Inspection Service permit (APHIS). Plants were also greenhouse tested at INRA, Bordeaux, France. One transgenic plum clone, C5, was identified as highly resistant to PPV through aphid- and graft- inoculation. This clone did not express PPV-CP and produced barely detectable levels of CP mRNA. Clones that did express the CP gene proved to be susceptible (Ravelonandro et al. 1997; Scorza et al. 2001). This suggested a resistance mechanism that was not protein-mediated. Molecular analyses have shown that C5 contains intact and partial copies of all three of the insert transgenes and the expression of the three genes is unequal. The PPV-CP transgene, produced only exceedingly low amounts of RNA transcript and no detectable PPV-CP (Scorza et al. 1994). The other two transgenes, *nptII* and *uidA* (GUS), express their respective proteins.

Further molecular analyses of the C5 PPV-CP transgene demonstrated a high level of RNA expression in the nucleus, yet low levels of PPV-CP mRNA in the cytoplasm, a complex multicopy insertion with partial insert copies, transgene methylation and confirmed the lack of PPV-CP expression (Scorza et al. 1994, 2001). These are all characteristics typical of post-transcriptional gene silencing (PTGS) (Depicker and van Montagu 1997). The PTGS mechanism of resistance in C5 was further confirmed by the detection of short interfering RNA (siRNA) homologous to the PPV-CP sequences (Hily et al. 2005). While production of the short (21nt) siRNA has been detected in non-transformed PPV-susceptible plums following inoculation with PPV, the resistant C5 produces both short (21nt) and long (24nt) species of siRNA. These studies lead to the conclusion that the high level virus resistance in the transgenic C5 results from the production of the long-sized class of siRNA (Hily et al. 2005; Kundu et al. 2008). Transgene silencing was the result of the activity of a hairpin DNA configuration that was apparently the result of a duplication and rearrangement during the insertion event (Scorza et al. 2010).

In 1993, a field trial of C5 and PPV-CP expressing transgenic plum lines was planted at the USDA-ARS Appalachian Fruit Research Station in Kearneysville, WV under US APHIS permit, although no PPV inoculations could be carried out due to quarantine restrictions. In order to evaluate PPV resistance, field tests were developed with research partners in Europe to test this resistant clone in areas where PPV was endemic. The field tests were conducted in Poland from 1996 to 2006, Spain from 1996 to 2012, Romania 1996–2006 (with a field test renewal granted in 2013), and Czech Republic 2002–2013. The European field tests clearly demonstrated the resistance of C5 to PPV infection through aphid vectors and by graft inoculation (Hily et al. 2004; Malinowski et al. 2006; Capote et al. 2008; Polák et al. 2008; Ravelonandro et al. 2002a; Zagrai et al. 2008a, 2008b). In all of these field tests no 'HoneySweet' trees were infected with PPV through natural aphid transmission. When grafted with PPV-infected budwood from susceptible hosts or grown on PPV-infected rootstock 'HoneySweet' trees have shown only mild symptoms in leaves localized near the point of grafting but no systemic virus spread was detected (Jarošová et al. 2010; Malinowski et al. 2006; Capote et al. 2008; Polák et al. 2008, 2012) and diminishing of localized symptoms was seen with time (Polák et al. 2008, 2012) While these graft-inoculation tests demonstrated that C5 is not immune to PPV, the virus remains confined close to the graft union with a low titer (Malinowski et al. 1998; Ravelonandro et al. 2007; Malinowski et al. 2006; Polák et al. 2008).

Both the complex structure of the inserted transgenes and the associated resistance to PPV have been found to be stable. The inserted DNA in C5 has been molecularly evaluated in both vegetatively propagated plants and seedlings and no changes have been detected (Scorza et al. 2001). In addition, expression of the resistance trait has been maintained for over 15 years of field trials with C5 clones grafted onto PPV susceptible rootstocks (Malinowski et al. 2006; Capote et al. 2008). The plants have remained free of systemic virus spread despite the very high inoculation pressure produced by grafting PPV infected material onto the transgenic shoots and grafting transgenic shoots onto infected rootstocks or in cases when the conventional rootstock became infected though aphid

Fig. 2 Harvest of 'HoneySweet' plums in test plot at Kearneysville, West Virginia, USA

feeding on root sucker shoots. 'HoneySweet' trees have been shown to be resistant to all strains of the virus tested (Ravelonandro et al. 2001a).

Based on evaluations of fruit quality and productivity 'HoneySweet' is not only protected against PPV but also has the attributes of fruit quality and yield that make is suitable for commercial production (http://naldc.nal.usda.gov/download/2915/PDF,acessed March 20, 2013) (Fig. 2). Nevertheless, if 'HoneySweet' could not be used in breeding to reliably transmit high level protection against PPV to its progeny, the impact of this variety would be limited. 'HoneySweet' was used as a parent in crosses to evaluate the transmission of the resistance trait. Although the transgene insert consisted of an inverted repeat with duplications of certain sequences, resistance to PPV was transmitted in a Mendelian fashion as a single locus dominant trait (Ravelonandro et al. 1998, 2001b, 2002b, 2011; Scorza et al. 1998). All the seedlings carrying the transgene insert were resistant to PPV (Scorza et al. 2001). This makes resistant hybrids easy to identify as the transgene insert in 'HoneySweet' provides molecular markers through use of polymerase chain reaction (PCR) of the transgene itself. The presence of the linked GUS gene in the insert also allows for simple and rapid selection of resistant seedlings using the GUS histological assay (Jefferson 1987).

The regulatory process

The commercial availability of 'HoneySweet' required regulatory approvals from the US APHIS, and the US Environmental Protection Agency (EPA). A voluntary submission to the US Food and Drug Administration (FDA) is also typically a part of the regulatory process for GE food products.

A substantial body of research had been developed in the years between the initial development of 'HoneySweet' and data submission to the US regulatory agencies which took place between 2003 and 2007. In addition to molecular characterization of the engineered plants, evaluation of resistance, and pomological studies, a series of risk assessment studies had been performed. These studies focused on the interaction of GE plums with PPV, aphid vectors, and with non-target insect species as well as gene flow. These investigations led to the findings that virus diversity was unaffected by PPV-CP transgenic plums (Capote and Cambra 2005; Capote et al. 2008; Zagrai et al. 2007, 2008c, 2011); no virus recombinants were produced (Zagrai et al. 2011; Fuchs et al. 2007); there were no measurable effects on aphid populations (Capote et al. 2008) or on other arthropods visiting the trees (Capote et al. 2007); no breakdown of PPV resistance in the presence of other *Prunus* viruses (*Prunus necrotic ringspot virus*, *Prune dwarf*, *Apple chlorotic ringspot virus*) (Polák et al. 2005a, 2005b, 2012; Zagrai et al. 2008a, b); and gene flow was quite low (http://www.aphis.usda.gov/brs/aphisdocs/04_26401p.pdf).

Pre-submission consultations with US regulatory agencies APHIS, FDA and EPA began in 2003 (Scorza et al. 2013). These consultations are not typical of many national regulatory systems but they are useful to applicants in preparing a submission that clearly addresses the regulatory requirements. APHIS has jurisdiction over the field testing of genetically engineered plants that contain plant pathogen genes or promoters. FDA has jurisdiction over GE plants used as food, and EPA regulates GE crop plantings of over ten acres for GE plants that produce molecules that protect plants against pests—protection against PPV in the case of 'HoneySweet'. An application for determination of non-regulatory status was submitted to APHIS in September 20, 2004. A revised application was submitted on March 13, 2006 based on APHIS comments. In May, 2006, the petition submitted to APHIS was posted on the internet for 60 days of public comment. APHIS received 1,725 comments, 1,708 were not in support of deregulation. A large number of comments of non-support appeared to have been duplicated, cut and pasted, at the urging of a single anti-GMO website. APHIS addressed the comments and a determination of non-regulated status was made on June 27, 2007. The result of an Environmental Assessment undertaken by APHIS was a Finding of No Significant Impact (http://www.aphis.usda.gov/brs/aphisdocs2/04_26401p_com.pdf).

The dossier provided to the FDA consisted of information pertaining to the food uses of plum, compositional analyses of 'HoneySweet' compared to non engineered plum varieties, and in silico analyses of allergenicity and antinutrient potentials. Several databases and alignment approaches were used including the Allermatch allergen finder (www.allermatch.org), 7 and 8 amino acid word

search using the same database, 80 amino acids sliding window alignment with the same database, and FASTA alignments done manually using the Codex Alimentarius guidelines which were used to create the Allermatch algorithms. The sequence was broken into 80 amino acid words and FASTA aligned with allergens (http://www.who.int/foodsafety/publications/biotech/en/ec_jan2001.pdf). The antinutrient potential of the insert sequences was evaluated using the NCBI antinutrient sequence data base. The submission to FDA was made on October 26, 2006 and was accepted on January 12, 2007. Following several requests for additional information or clarifications a communication was received from FDA on January 16, 2009 stating that, "Based on the safety and nutritional assessment USDA-ARS conducted, it is the understanding of FDA that USDA-ARS has concluded that plums derived from the new variety are not materially different in composition, safety, and other relevant parameters from plums currently on the market and that the genetically engineered plum line C5 does not raise issues that would require premarket review of, or approval by, FDA." This letter indicated the completion of the FDA review.

A dossier was submitted to EPA in June, 2007. The initial EPA scientific review resulted in a request for additional information and/or clarifications and responses were submitted in July 2008. On October 29, 2008 EPA published in the Federal Register (73 FR 64325) a Notice of Receipt announcing an application to register a pesticide product containing a new active ingredient not included in any currently registered pesticide product (the PPV-CP gene). Four public comments were received during a 30 days comment period following the publication of the notice, all favorable.

EPA published a notice of filing of the petition in the Federal Register on November 14, 2008 (73 FR 67512) and the public was given a 30 days comment period. EPA received no comments on this notice. EPA required an independent laboratory validation of the proposed method for detecting the transgene in 'HoneySweet' leaves. The leaf samples along with the detection method were sent out for third party validation. On April 1, 2010 the draft registration was published on the web (http://www.regulations.gov/#!docketDetail;D=EPA-HQ-OPP-2008-0742, accessed March 21, 2013) with a comment period ending on April 30, 2010. Sixty-six comments were received; sixty-four were highly supportive of registration, including some questioning the need for registration and the classification of 'HoneySweet plum as a biopesticide. These questions were raised with regard to the fact that the mechanism of resistance does not produce a plant incorporated protectant since no CP is produced and DNA or nucleic acids have never been considered alone to be pesticidal substances. The labeling of trees as pesticidal was also brought into question. It was suggested that mandatory labeling of 'HoneySweet' trees and propagative material as pesticidal (fruit would not be labeled) would cause substantial damage to the market for 'HoneySweet' and sets a precedent for future transgenic virus resistant crops to be treated "in the same unscientific and irrational manner." (for specific comments cited see http://www.regulations.gov/#!docketDetail;D=EPA-HQ-OPP-2008-0742, accessed March 21, 2013). EPA issued an unconditional Section 3 registration on August 8, 2011. At this point 'HoneySweet' had successfully completed the US regulatory process, the first woody perennial tree fruit to have done so.

Future prospects for PPV resistance through GE

A number of other programs world-wide have produced PPV resistant herbaceous species or plums using RNAi technology (see review of Ilardi and Di Nicola-Negri 2011). Much of the work has involved hairpin constructs of the PPV-CP gene (Hily et al. 2007; Di Nicola-Negri et al. 2010; Dolgov et al. 2010; Mikhailov and Dolgov 2007; Wang et al. 2009).

At this point in the research and development of RNAi technology for PPV resistance additional research in the transformation and regeneration of Prunus species is necessary for the broader application of this technology. Although some successes have been reported (Dai et al. 2004; Damiano et al. 2007; Espinosa et al. 2006; Gentile et al. 2002; Padilla et al. 2006; Petri et al. 2008; Ramesh et al. 2006; Song and Sink 2006; Tian et al. 2007; Urtubia et al. 2008) there is still much work to be done to develop reliable, productive *Prunus* transformation systems. The ability to transform and regenerate from clonal material versus seed-derived explants would be an important step and allow the rapid incorporation of virus resistance into existing varieties. Dolgov et al. (2010) report a successful example of developing PPV-CP RNAi plums from leaf explants of the cultivar 'Startovaya'.

Silencing of multiple targets in the viral genome would provide broader-based horizontal resistance. Work with a number of targets such as P1, HC-Pro, P3 (Di Nicola-Negri et al. 2010) and with artificial micro RNAs (Ravelonandro et al. 2013) has demonstrated the feasibility of this approach. RNAi silencing of the host factor eIF(iso)4E in plum presents an example of an alternative RNAi target for developing host plant resistance to PPV (Wang et al. 2013). Further refinements such as combining RNAi-induced resistance with other resistance mechanisms, natural or GE, can provide a further level of security in terms of the durability of protection.

Conclusions

The approval of 'HoneySweet' in the US has marked a milestone for the practical application of GE technology

for the improvement of temperate fruit trees. We have, through international cooperation between public institutions in six countries, utilized genetic engineering to develop resistance to a major pathogen of a woody perennial fruit crop, taking the project from concept to a product that has passed regulatory scrutiny in the US.

We are currently hybridizing 'HoneySweet' with a range of cultivars from different *P. domestica* growing areas to develop new adapted resistant cultivars. These new cultivars derived from 'HoneySweet' will not require additional regulatory approval in the US. We are accelerating the breeding process using 'FasTrack' breeding which utilizes the Poplar *Flowering Locus T (PtFT)* gene (Böhlenius et al. 2006; Zhang et al. 2010) (Fig. 3). *PtFT* transgenic early flowering parental lines reduce the generation time from 4 years to 1 year (Srinivasan et al. 2012; http://ucanr.edu/sites/fastrack/, accessed March 19, 2013). We have also produced new PPV resistant plum lines silencing CP (Hily et al. 2007) and other PPV genes and these plants are currently under test.

We believe that 'HoneySweet' presents an important step in the utilization of genetic engineering to help solve major virus diseases of fruit trees. The approach documented by the research and development effort used to produce 'HoneySweet' has been shown to be effective, durable, and safe as a food and in the environment (CERA 2012).

Regulatory and acceptance barriers to the release of GE virus resistant fruit crops exist and these are particularly evident in the EU. Over the past 15 years we have conducted a number of studies on environment risks and food safety in the EU and these studies have consistently demonstrated the safety of 'HoneySweet' plum, and by extension the technology used in its development. Current technologies have allowed DNA and small RNA sequencing of 'HoneySweet' (Callahan et al. 2010; C. Dardick, A. Callahan, T. Malinowski, unpublished) and these studies confirm prior safety assessments. Gene flow studies conducted for over a decade demonstrate the potential for coexistence of GE and conventional plums (Scorza et al. submitted). We look forward to regulatory approvals of 'HoneySweet' in other regions affected by PPV, and the development, testing and regulatory approval of new GE PPV-resistant cultivars from other genetic improvement programs. The efficacy, durability, and safety of 'HoneySweet' plum in the protection against PPV establish the practical advantages of RNAi technology for developing virus resistant crops and warrant its further application for protecting agricultural production. To accomplish this, research must move from the laboratory to the field and through the regulatory process. This is the real challenge to the technology.

Fig. 3 Poplar *Flowering Locus T (PtFT)* transgenic early flowering *P. domestica* plum line in the greenhouse hybridized with 'HoneySweet' for rapid incorporation of the 'HoneySweet' resistance insert into new *P. domestica* germplasm and cultivars

Acknowledgments The authors wish to acknowledge the following persons and organizations that have contributed scientific expertise or otherwise supported the 'HoneySweet' research and development effort: A. Atanassov, J. Dougherty, L. Fortis, L. Georgi, J. Kumar, Black Sea Biotechnology Association, USDA-Foreign Agricultural Service, G. Llacer (Spain), B. Zawadzka (Poland) and N. Minoiu (Romania) who initiated 'HoneySweet' field tests in Europe. The field test in Spain was initiated under EU-BIOTECH FP4-"Risk assessment of genetically engineered woody plants expressing virus coat protein gene" (1996–1999). Studies undertaken in the Czech Republic were supported by the Czech Ministry of Agriculture, Grants No. QI101A123, and No. 0002700604 and in Romania by EU contract "TRANSVIR"QLK3-CT-2002-02140 and by the Romanian Research Ministry, contract 37/2003.

References

Acuna R (1993) Outbreaks of *Plum pox virus* in Chile. Eur. Mediterr. Plant Prot Org Conf *Plum pox virus*, August 5–8, Bordeaux, France

Anonymus (1970) Situation phytosanitaire de le sharka. Publications OEPP/EPPO, Série B 66

Atanassoff D (1932/1933) Plum pox. A new virus disease. Yearbook, vol 11. Fac Agric Univ, Sofia, pp 49–69

Badenes ML, Llácer G (2006) Breeding for resistance: breeding for *Plum pox virus* resistant apricots (*Prunus armeniaca* L.) in Spain. Bull OEPP/EPPO Bull 36:323–326

Barba M, Hadidi A, Candresse T, Cambra M (2011) *Plum pox virus*. In: Hadidi A, Barba M, Candresse T, Jelkmann W (eds) Virus and virus-like disease of pome and stone fruits. APS Press, St. Paul, pp 185–197

Beachy RN, Loesch-Fries S, Tumer NE (1990) Coat-protein-mediated resistance against virus infection. Ann Rev Phytopathol 28:451–474

Blattný C (1930) Comments on virus and related diseases of plants I. Ochr Rostl 10:130–138 (in Czech)

Böhlenius H, Huang T, Charbonnel-Campaa L, Brunner AM, Jansson S, Strauss SH, Nilsson O (2006) *CO/FT* regulatory module

controls timing of flowering and seasonal growth cessation in trees. Science 312:1040–1043
Callahan A, Dardick C, Scorza R (2010) Utilization of next-generation sequencing for analyzing transgenic insertions in plum: a risk assessment study. HortScience 45:S6 (Abstr.) http://ashs.org/downloads/HortScience45(8)supplement.pdf
Cambra M, Capote N, Myrta A, Llácer G (2006) *Plum pox virus* and the estimated costs associated with sharka disease. Bull OEPP/EPPO Bull 36:202–204
Canova A, Giunchedi L, Credi R, Albertini A, Nicolli C (1977) Sharka or plum pox of apricot and plum in Italy. Frutticoltura 39:17–23 (in Italian)
Capote N, Cambra M (2005) Variability of *Plum pox virus* populations in PPV-resistant transgenic and non-transgenic plums. Phytopathol Pol 36:107–113
Capote N, Pérez-Panádes J, Monzó C, Carbonell EA, Urbaneja A, Scorza R, Ravelonandro M, Cambra M (2007) Evaluación del riesgo ambiental de cultivo en campo de ciruelos transgénicos sensibles y resistentes a *Plum pox virus*. ITEA 103:156–167
Capote N, Pérez-Panádes J, Monzó C, Carbonell EA, Urbaneja A, Scorza R, Ravelonandro M, Cambra M (2008) Assessment of the diversity of *Plum pox virus* and aphid populations on transgenic European plums under Mediterranean conditions. Transgenic Res 17:367–377
CERA (2012) GM Crop Database. Center for environmental risk assessment (CERA), ILSI Research Foundation, Washington D.C. http://cera-mc.org/index.php?action=gm_crop_database
Crescenzi A, Nuzzaci M, Piazzolla P, Levy L, Hadid A (1995) *Plum pox virus* (PPV) in sweet cherry. Acta Hortic 386:219–225
Cropley R (1968) The identification of plum pox (sharka) virus in England. Plant Pathol 17:66–70
Dai WH, Jacques V, Walla JA, Cheng ZM (2004) Plant regeneration of chokecherry (*Prunus virginiana* L.) from in vitro leaf tissues. J Environ Hortic 22:225–228
Dal Zotto A, Ortego JM, Raigón JM, Caloggero S, Rossini M, Ducasse CA (2006) First report in Argentina of *Plum pox virus* causing sharka disease in *Prunus*. Plant Dis 90:523
Damiano C, Gentile A, Monticelli S, Kondakova V, Todorovska E, Kamenova I, Badjiakov I, Atanassov A, Scorza R (2007) Improving regeneration and transformation for resistance to sharka in *Prunus*. Acta Hortic 738:583–587
Damsteegt VD, Scorza R, Stone AL, Schneider WL, Webb K, Demuth M, Gildow FE (2007) *Prunus* host range of *Plum pox virus* (PPV) in the United States by aphid and graft inoculation. Plant Dis 91:18–23
Demetriades SD, Castimbas C (1968) Attaques et nouveaux ennemis signalés (sharka). Bull Phytosanitaire FAO 16:10–11 (in French)
Depicker A, Van Montagu M (1997) Post-transcriptional gene silencing in plants. Curr Opin Cell Biol 9:373–382
Di Nicola-Negri E, Tavazza M, Salandri L, Ilardi V (2010) Silencing of *Plum pox virus* 5"UTR/P1 sequence confers resistance to a wide range of PPV strains. Plant Cell Rep 29:1435–1444
Dolgov S, Mikhaylov R, Serova T, Shulga O, Firsov A (2010) Pathogen-derived methods for improving resistance of transgenic plums (*Prunus domestica* L.) for *Plum pox virus* infection. Julius-Kuhn-Arch 427:133–140
Dondini L, Lain O, Vendramin V, Rizzo M, Vivoli D, Adami M, Guidarelli M, Gaiotti F, Palmisano F, Bazzoni A et al (2011) Identification of a QTL for resistance to *plum pox virus* strains M and D in Lito and Harcot apricot cultivars. Mol Breed 27:289–299
Dosba F, Orliac S, Maison P, Massione G, Audergon JM (1992) Evaluation of resistance to *plum pox virus* in apricot trees. Acta Hortic 309:211–219
Dunéz J (1987) Plum pox disease of stone fruit trees. FAO, Rome
Espinosa AC, Pijut PM, Michler CH (2006) Adventitious shoot regeneration and rooting of *Prunus serotina* in vitro cultures. HortScience 41:193–201
Ferreira SA, Pitz KY, Manshardt R, Zee F, Fitch M, Gonsalves D (2002) Virus coat protein transgenic papaya provides practical control of papaya ringspot virus in Hawaii. Plant Dis 86:101–105
Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391(806):811
Fitch MM, Manshardi RM, Gonsalves D, Slightom JL, Sanford JC (1990) Stable transformation of papaya via microprojectile bombardment. Plant Cell Rep 9:189–194
Fuchs M, Cambra M, Capote N, Jelkmann W, Kundu J, Laval V, Martelli GP, Minafra A, Petrovic N, Pfieffer P, Pompe-Nocak M, Ravelonandro M, Sldarelli P, Stussi-Garaud C, Vigne E, Zagrai I (2007) 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. J Plant Pathol 89:5–12
Gentile A, Monticelli S, Damiano C (2002) Adventitious shoot regeneration in peach [*Prunus persica* (L.) Batsch]. Plant Cell Rep 20:1011–1016
Gildow F, Damsteegt V, Stone A, Schneider W, Luster D, Levy L (2004) Plum pox in North America: identification of aphid vectors and a potential role for fruit in virus spread. Phytopathology 94:868–874
Glasa M, Hricovsky L, Kudela O (1999) Evidence for non-transmission of *Plum pox virus* by seed in infected plum and myrabolan. Biologia Bratisl 54:481–484
Gonsalves D (1998) Control of papaya ringspot virus in papaya: a case study. Ann Rev Phytopathol 36:415–437
Hamilton A, Baulcombe D (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:950–952
Hartmann W, Neumüller M (2006) Breeding for resistance: breeding for *Plum pox virus* resistant plums (*Prunus domestica* L) in Germany. Bull OEPP/EPPO Bull 36:332–336
Hartmann W, Petruschke M (2000) Sharka resistant plums and prunes by utilization of hypersensitivity. Acta Hortic 538:392–395
Hily JM, Scorza R, Malinowski T, Zawadska B, Ravelonandro M (2004) Stability of gene silencing-based resistance *to Plum pox virus* in transgenic plum (*Prunus domestica* L.) under field conditions. Transgenic Res 13:427–436
Hily JM, Scorza R, Webb R, Ravelonandro M (2005) Accumulation of the long class of siRNA is associated with resistance to *Plum pox virus* in transgenic woody perennial plum tree. Mol Plant Microbe Interact 8:794–799
Hily JM, Ravelonandro M, Damsteegt V, Bassett C, Petri C, Liu Z, Scorza R (2007) *Plum pox virus* coat protein gene intron-hairpin-RNA (ihpRNA) constructs provide resistance to *Plum pox virus* in *Nicotiana benthamiana* and *Prunus domestica*. J Am Soc Hortic Sci 132:850–858
Ilardi V, Di Nicola-Negri E (2011) Genetically engineered resistance to *Plum pox virus* infection in herbaceous and stone fruit hosts. GM Crops 2:24–33
Isac M, Preda S, Marcu M (1998) Aphid species—vectors of *plum pox virus*. Acta Virol 4:233–234
James D, Glasa M (2006) Causal agent of sharka disease: new and emerging events associated with *Plum pox virus* characterization study. Bull OEPP/EPPO Bull 36:247–250
James D, Varga A (2005) Nucleotide sequence analysis of *Plum pox virus* isolate W3174: evidence of a new strain. Virus Res 110:50–143
James D, Varga A (2011) Genetic diversity of Canadian isolates of *Plum pox virus* and its significance. Acta Hortic 899:29–38

James D, Varga A, Thompson D, Hayes S (2003) Detection of a new and unusual isolate of Plum pox potyvirus in plum (*Prunus domestica*). Plant Dis 87:1119–1124

Jarošová J, Gadiou S, Polák J, Ravelonandro M, Scorza R, Kumar JK (2010) Evaluation of transgenic *Prunus domestica* L., clone C5 resistance to *Plum pox virus*. Julius-Kuhn Arch 427:330–333

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387–405

Jordovic M (1968) Effect of sources of infection on epidemiology of sharka (plum pox) virus disease. Tagungsber. DL DDR Berlin 97:301–308

Josifović M (1937) Mosaic of plum. Arch Minister Poljopriv 4:131–143 (in Serbochorv.)

Kalayshan JA, Bilkey ND, Verderevskaya TD, Rubina EV (1994) *Plum pox virus* in sour cherry in Moldova. Bull OEPP/EPPO Bull 24:645–649

Kamenova I, Scorza R, Ravelonandro M, Callahan A, Paunovic S, Zagrai I, Dorokhov D (2010) Blume Y (2010) Case study: reducing the harmful impacts of *Plum pox virus* through the use of biotechnology. In: Golikov A, Atanassov A (eds) Regional consensus documents on environmental risk and economic assessment of genetically modified crops. Black Sea Biotechnology Association, Infoprint, pp 127–151

Karayiannis I, Mainou A, Tsaftaris A (1999) Apricot breeding in Greece for fruit quality and resistance to *plum pox virus*. Acta Hortic 488:111–117

Kegler H, Hartmann W (1998) Present status of controlling conventional strains of *Plum pox virus*. In: Plant virus control. American Phytopathology Society, 3340, Pilot Knob Road, St. Paul, Minnesota pp 616–628

Kegler H, Schade C (1971) *Plum pox virus*. CMI/AAB Descr Plant Vir 70:1–4

Kegler H, Verderevskaja TD, Bivol TF (1985) Detection of different resistance types of plants against sharka virus (*Plum pox virus*). Arch für Phytopath und Pflanzeschutz 21:339–346

Kegler H, Hartmann W, Fuchs E, Gruntzig M (1995) Different host reaction can involve field resistance of plum genotype to *plum pox virus*. Acta Hortic 386:306–310

Kollerova E, Novakova S, Subr Z, Glasa M (2006) *Plum pox virus* mixed infection detected on apricot in Pakistan. Plant Dis 90:1108

Kundu JK, Briard P, Hily JM, Ravelonandro M, Scorza R (2008) Role of the 25–26 nt siRNA in the resistance of transgenic Prunus domestica graft inoculated with *plum pox virus*. Virus Genes 36:215–220

Kunze L, Krczal H (1971) Transmission of sharka virus by aphids. In: Proceedings of the 8th European Symposium of Fruit Tree Virus Diseases, Institut National de la Recherche Agronomique, Paris, pp 255–260

Labonne G, Lauriaut F, Yvon M, Quiot JB, Avinent L, Llacer GL (1995) Aphids as potential vectors of *Plum pox virus*. Comparison of methods of testing and epidemiological consequences. Acta Hortic 386:207–218

Lalli DA, Abbott AG, Zhebentyayeva TN, Badenes ML, Damsteegt V, Polák J, Krška B, Salava J (2008) A genetic linkage map for an apricot (*Prunus armeniaca* L.) BC1 population mapping *plum pox virus* resistance. Tree Genet Genomes 4:481–493

Lambert P, Dicentra F, Rubio M, Audergon JM (2007) QTL analysis of resistance to sharka disease in the aprocot (*Prunus armeniaca* L.) 'Polanais' x 'Stark Early Orange' F1 progeny. Tree Genet Genomes 3:299–309

Levy L, Damsteegt V, Welliver R (2000) First report of *Plum pox virus* (Sharka disease) in *Prunus persicae* in the United States. Plant Dis 84:202

Lichtenegger L, Neumuller M, Treutter D, Hartmann W (2010) The inheritance of the hypersensitivity resistance of European plum (Prunus domestica L.) against the *Plum pox virus*. Julius-Künh Arch 427:327–329

Ling KS, Namba S, Gonsalves C, Slightom JL, Gonsalves D (1991) Protection against detrimental effects of *Potyvirus* infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. Biotechnology 9:752–758

Llácer G (2006) Hosts and symptoms of *Plum pox virus*: herbaceous hosts. Bull OEPP/EPPO Bull 36:227–228

Llácer G, Cambra M (2006) Hosts and symptoms of *Plum pox virus*: fruiting Prunus species. Bull OEPP/EPPO Bull 36:219–221

Llácer G, Cambra M, Lavina A (1985) Detection of *plum pox virus* in Spain. Bull OEPP/EPPO Bull 15:325–329

Louro D, Corvo LM (1985) Occurrence of sharka in Portugal. Acta Hortic 193:183

Maejima K, Hoshi H, Hashimoto M, Himeno M, Kawanishi T, Komatsu K, Yamaji Y, Hamamoto H, Namba S (2010) First report of *Plum pox virus* infecting Japanese apricot (*Prunus mume* Sieb. Et Zucc) in Japan. J Gen Plant Pathol 76:226–231

Malinowski T, Zawadzka B, Ravelonandro M, Scorza R (1998) Preliminary report on the apparent breaking of resistance of transgenic plum by chip budding inoculation of *Plum pox virus* PPV-S. Acta Virol 42:241–243

Malinowski T, Cambra M, Capote N, Zawadska B, Gorris MT, Scorza R, Ravelonadro M (2006) Field trials of plum clones transformed with the *Plum pox virus* coat protein (PPV-CP) gene. Plant Dis 90:1012–1018

Marandel G, Salava J, Abbott A, Candresse T, Decroocq V (2009) Quantitative trait loci meta-analysis of *Plum pox virus* resistance in apricot (Prunus armeniaca L.): new insights on the organization and the identification of genomic resistance factors. Mol Plant Pathol 10:347–360

Maroquin C, Rassel A (1976) Caractérisation du *plum pox virus* sur un prunier en Belgique. Parasitica 32:93–108 (in French)

Meijneke CAR (1967) Sharka in plums and peaches. Fruitteelt 57:251–252 (in Dutch)

Mendonca D, Lopes MS, Laimer de Camara Machado M, Da Camara Machado A (1997) Diagnosis of viral diseases in stone fruits cultivated in the Azorean islands Terceira and Graciosa. In: Proceedings 17th Internat Symp Virus Virus-like Dis Temperate Fruit Crops. Bethesda: 40 (Abstr.)

Mikhailov RV, Dolgov SV (2007) Transgenic plum (*Prunus domestica* L.) plants obtained by Agrobacterium-mediated transformation of leaf explants with various selective agents. Acta Hortic 738:613–623

Milusheva S, Gercheva P, Bozhkova V, Kamenova I (2008) Experiments on transmission of *plum pox virus* through *Prunus* seeds. J Plant Pathol 90:S1.23–S1.26

Minoiu N (1997) Plum diseases and pests. In: Cociu I, Botu I, Minoiu NI, Modoran I (eds) The Plum. Editure Conphys, Pitesti, pp 343–374

Minoiu N, Maxim A, Vladianu D, Platon I, Balaci R (1998) New results concerning the *plum pox virus* epidemiology and resistance of plum cultivars, hybrids and rootstocks. Acta Virolgica 42:244–247

Moreno A, Fereres A, Cambra M (2009) Quantitative estimation of *Plum pox virus* targets acquired and transmitted by a single *Myzus persicae*. Arch Virol 154:1391–1399

Navratil M, Safarova D, Karesova R, Petrzik K (2005) First evidence of *Plum pox virus* on apricot trees in China. Plant Dis Note 89:338

Nemchinov L, Hadidi A (1996) Characterization of the sour cherry strain of *Plum pox virus*. Phytopathology 86:575–580

Neumüller M, Hartmann W (2008) The phenotypically quantitative nature of hypersensitivity of European plum (*Prunus domestica* L.) against the *Plum pox virus* and its description using the hypersensitivity index. Hortic Sci (Prague) 35:50–64

Neumüller M, Hartmann W, Petruschke M, Treutter D (2010) The hypersensitivity resistance of European plum to the *Plum pox virus* and its potential impact on the epidemiology of the virus. Julius-Kühn Arch 427:147–150

OEPP/EPPO (1983) Data sheets on quarantine organisms No. 96, *Plum pox virus*. Bull OEPP/EPPO Bull 13 (1)

OEPP/EPPO Bull (2006) A review of *Plum pox virus*. In: Capote N, Cambra M, Llacer G, Petter F, Platts LG, Roy AS, Smith IM (eds) Blackwell Publishing Los Angeles, CA, pp 201–349

Olmos A, Capote N, Candresse T (2006) Detection and characterization of *Plum pox virus*: molecular methods. Bull OEPP/EPPO Bull 36:262–266

Padilla IMG, Golis A, Gentile A, Damiano C, Scorza R (2006) Evaluation of transformation in peach *Prunus persica* explants using green fluorescent protein (GFP) and beta-glucuronidase (GUS) reporter genes. Plant Cell Tissue Organ Cult 84:309–314

Papingyi A (1965) Lija në formë unaze e kumbullës. Plum ring pox on plum. Bull Shke Bujq, Tirane 2:31–32 (in Albanian)

Pasquini G, Barba M (2006) The question of seed transmissibility of *Plum pox virus*. Bull OEPP/EPPO Bull 36:287–292

Pasquini G, Simeone AM, Conte L, Barba M (1998) Detection of *Plum pox virus* in apricot seeds. Acta Virol 42:260–263

Pasquini G, Simeone AM, Conte L, Barba M (2000) RT-PCR evidence of the non-transmission through seed of *Plum pox virus* strains D and M. J. Plant Pathol 82:221–226

Petri C, Webb K, Hily J-M, Dardick C, Scorza R (2008) High transformation efficiency in plum (*Prunus domestica* L.): a new tool for functional genomics studies in *Prunus spp*. Mol Breeding 22:581–591

Pieniazek SA (1962) Survey on fruit tree virus diseases present in Poland. In: Proceedings 5th European symposium fruit tree virus diseases. Bologna

Polák J, Jarošová J (2012) Susceptibility of plum trees cv. 'Jojo' to a Czech isolate of *Plum pox virus* strain D. Can J Plant Pathol 34:283–287

Polák J, Komínek P (2001) Further evidence of the non-transmissibility of *Plum pox virus* through seeds: myrobalan (*Prunus cerasifera*). Phytopathol Pol 20:39–46

Polák J, Komínek P (2012) Biological evidence for practical immunity of apricot cultivar Harlayne to *Plum pox virus*. Plant Protect Sci 48:143–148

Polák J, Oukropec I, Chod J (1992) The production of sharka free propagating material of apricots and peaches in Czech Republic. Acta Hortic 309:139–142

Polák J, Pívalová J, Jokeš M, Svoboda J (2005a) Prune cv. Jojo resistance to different strains of *Plum pox virus*. Plant Protect Sci 41:47–51

Polák J, Pívalová J, Jokeš M, Svoboda J, Scorza R, Ravelonandro M (2005b) Preliminary results of interactions of *Plum pox* (PPV), *Prune dwarf* (PDV), and *Apple chlorotic leafspot* (ACLSV) *viruses* with transgenic plants of plum, *Prunus domestica* L. clone C-5 grown in an open field. Phytopathol Pol 36:115–122

Polák J, Pívalová J, Kumar J, Jokeš M, Scorza R, Ravelonandro M (2008) Behaviour of transgenic *Plum pox virus*-resistant *Prunus domestica* L. clone C5 grown in the open field under a high and permanent infection pressure of the PPV-REC strain. J Plant Pathol 90:33–36

Polák J, Kumar J, Krška B, Ravelonandro M (2012) Biotech/GM crops in horticulture: plum cv. Honeysweet resistant to *Plum pox virus*. Plant Protect Sci 48:S43–S48

Ramesh SA, Kaiser BN, Franks T, Collins G, Sedgley M (2006) Improved methods in *Agrobacterium*-mediated transformation of almond using positive (mannose/*pmi*) or negative (kanamycin resistance) selection-based protocols. Plant Cell Rep 25:821–828

Ravelonandro M, Monsion M, Teycheney PY, Delbos R, Dunez J (1992) Construction of a chimeric viral gene expressing *Plum pox virus* coat protein. Gene 120:167–173

Ravelonandro M, Scorza R, Bachelier JC, Labonne G, Levy L, Damsteegt V, Callahan AM, Dunez J (1997) Resistance of transgenic *Prunus domestica* to *Plum pox virus* infection. Plant Dis 81:1231–1235

Ravelonandro M, Scorza R, Renaud R, Salesses G (1998) Transgenic plums resistant to *Plum pox virus* infection and preliminary results of cross-hybridization. Acta Hortic 478:67–71

Ravelonandro M, Briard P, Scorza R (2001) Significant resistance of transgenic plums against the four serotypes of *plum pox* potyvirus. Acta Hortic 550:431–435

Ravelonandro M, Scorza R, Minoiu R, Zagrai I, Platon I (2002a) Field tests of transgenic plums in Romania. Sănătatea Plant 6:16–17

Ravelonandro M, Briard P, Monsion M, Scorza R (2002b) Stable transfer of the *Plum pox virus* (PPV) capsid transgene to seedlings of two French cultivars 'Prunier d'Ente 303' and 'Quetsche 2906', and preliminary results of PPV challenge assays. Acta Hortic 577:91–96

Ravelonandro M, Scorza R, Kundu J, Briard P, Monsion M (2007) The effect of co-infecting viruses on transgenic *Plum pox virus* resistant plums (*Prunus domestica*). Acta Hortic 738:653–656

Ravelonandro M, Scorza R, Briard P, Lafargue B, Renaud R (2011) Inheritance of silencing in transgenic plums. Acta Hortic 899:139–144

Ravelonandro M, Briard P, Hily JM (2013) RNAi to silence the *plum pox virus* genome. Acta Hortic 974:165–174

Ravelonanrdo M, Briard P, Renaud R, Scorza R (2001) Transgene-based resistance to *Plum pox virus* (Sharka disease) is transferred through inter-specific hybridization in *Prunus*. Acta Hortic 546:569–574

Roy AS, Smith IM (1994) Plum pox situation in Europe. Bull OEPP/EPPO Bull 24:515–523

Sahtiyanci S (1969) Virus de la sharka chez le prunier. Bull Phytosanitaire FAO 17:69 (in French)

Salava J, Wang Y, Krška B, Polák J, Komínek P, Miller WR, Dowler LW, Reighard LG, Abbott GA (2001) Identification of molecular markers linked to resistance of apricot (*Prunus armeniaca* L.) to *Plum pox virus*. In: Plant and Animal Genome IX Conference, San Diego, USA: http://www.intl-pag.org/pag/10/abstracts/P5h_01.html

Salava J, Wang Y, Krška B, Polák J, Komínek P, Miller W, Dowler W, Reighard GL, Abbott AG (2002) Identification of molecular markers linked to resistance of apricot (*Prunus armeniaca* L.) to *plum pox virus*. J Plant Dis Protect 109:64–67

Sanford JC, Johnston SA (1985) The concept of parasite-derived resistance-deriving resistance genes from the parasite's own genome. J Theor Biol 113:395–405

Schuch K (1957) Virus diseases and similar phenomena in fruit crops. Mitt. Biologischen Bundesanstalt für Land- und Forstwirtschaft 88:126–128 (in German)

Scorza R, Ravelonandro M, Callahan AM, Cordts JM, Fuchs M, Dunez J, Gonsalves D (1994) Transgenic plums (*Prunus domestica*) express the *Plum pox virus* coat protein gene. Plant Cell Rep 14:18–22

Scorza R, Levy L, Damsteegt V, Yepes M, Cordts J, Hadidi A, Slightom J, Gonsalves D (1995) Transformation of European plum (*Prunus domestica* L.) with the papaya ringspot virus coat protein gene and reaction of transgenic plants to inoculation with *plum pox virus*. J Am Soc Hortic Sci 120:943–952

Scorza R, Callahan AM, Levy L, Damsteegt V, Ravelonandro M (1998) Transferring potyvirus coat protein gene through hybridization of transgenic plants to produce *Plum pox virus* resistant plums (*Prunus domestica*). Acta Hortic 472:421–427

Scorza R, Callahan A, Levy L, Damsteegt V, Webb K, Ravelonandro M (2001) Post-transcriptional gene silencing in *Plum pox virus*

resistant transgenic European plum containing the *plum pox* potyvirus coat protein gene. Transgenic Res 10:201–209

Scorza R, Georgi L, Callahan A, Petri C, Hily J-M, Dardick C, Damsteegt V, Ravelonandro M (2010) Hairpin *Plum pox virus* coat protein (hpPPV-CP) structure in 'HoneySweet' C5 plum provides PPV resistance when genetically engineered into plum (*Prunus domestica*) seedlings. Julius-Kühn Arch 427:141–146

Scorza R, Callahan A, Ravelonandro M, Braverman M (2013) Regulation of agricultural biotechnology: The United States and Canada. In: Wozniak CA, McHughen A (eds) Development and regulation of the *Plum pox virus* resistant transgenic plum 'HoneySweet'. Springer, Dordrecht, pp 269–280

Song G-Q, Sink KC (2006) Transformation of Montmorency sour cherry (*Prunus cerasus* L.) and Gisela 6 (*P. cerasus* x *P. canescens*) cherry rootstock mediated by *Agrobacterium tumefaciens*. Plant Cell Rep 25:117–123

Soriano JM, Domingo ML, Zuriaga E, Romero C, Zhebentyayeva T, Abbott AG, Badenes ML (2012) Identification of simple sequence repeat markers tightly linked to *plum pox virus* resistance in apricot. Mol Breeding 30:1017–1026

Spiegel S, Kovalenko E, Varga A, James D (2004) Detection and partial molecular characterization of two *Plum pox virus* isolates from plum and wild apricot in southeast Kazakhstan. Plant Dis 88:973–979

Srinivasan C, Dardick C, Callahan A, Scorza R (2012) Plum (Prunus domestica) trees transformed with poplar *FT1* result in altered architecture, dormancy, requirement, and continuous flowering. PLoS ONE 7:e40715 doi/10.1371/journal.pone.0040715

Szczygieł A (1962) Pojawienie się w Polsce groźnej choroby wirusowej szarki *Prunus virus*-7. [The appearence of dangerous sharka virus disease - *Prunus virus*-7, in Poland] Ochrona Roślin 1–2:14–17

Szirmai J (1948) Virus disease of apricot. Magyar Bor és Gyümölcs 3:7–8 (in Hungarian)

Thakur PD, Bhardwaj SV, Garg ID, Kihore-Khosla SDR, Khosla K (1994) *Plum pox virus* on stone fruits in India—a new record. Plant Dis Res 9:100–102

Thompson D, McCann M, MacLeod M, Lye D, Green M, James D (2001) First report of *Plum pox potyvirus* in Ontario. Canada Plant Dis 85:97

Thompson D, Varga A, De Costa H, Birch C, Glasa M, James D (2009) First report of *Plum pox virus* recombinant strain on *Prunus* spp. in Canada. Plant Dis 93:674

Tian L, Wen Y, Jayasankar S, Sibbald S (2007) Regeneration of *Prunus salicina* Lindl (Japanese plum) from hypocotyls of mature seeds. Vitro Cell Dev Biol Plant 43:343–347

Urtubia C, Devia J, Castro A, Zamora P, Aguirre C, Tapia E, Barba P, Dell'Orto P, Moynihan MR, Petri C, Scorza R, Prieto H (2008) *Agrobacterium*-mediated genetic transformation of *Prunus salicina*. Plant Cell Rep 27:1333–1340

van Oosten HJ (1971) The isolation of Sharka (plum pox) virus from leaves and fruits of plum with herbaceous plants. Neth J Plant Pathol 76:99–103

Verderevskaya T (1965) Virus diseases of fruit trees in Moldavia. Zascita Bilya 16:509–512

Vukovits G (1961) Presence of virus diseases in Austrian orchards. Tidskrift for Planteavl 65 (special issue) pp 204–210 (in German)

Wallis CM, Fleischer SJ, Luster D, Gildow FE (2005) Aphid (Hemiptera: Aphididae) species composition and potential aphid vectors of *plum pox virus* in Pennsylvania peach orchards. J Econ Entomol 98:1441–1450

Wang A, Tian L, Huang TS, Brown DCW, Svircev AM, Stobbs LW, Miki B, Sanfaçon H (2009) The development of genetic resistance to *plum pox virus* in transgenic *Nicotiana benthamiana* and *Prunus domestica*. Acta Hortic 839:665–672

Wang X, Kohalmi SE, Svircev A, Wang A, Sanfaçon H et al (2013) Silencing of the host factor eIF(iso)4E gene confers *plum pox virus* resistance in plum. PLoS ONE 8:e50627. doi:10.1371/journal.pone.0050627

Zagrai L, Zagrai I (2008) Non-transmission of *Plum pox virus* D and Rec strains through seeds in plum. Bull UASVM Hort 65:523

Zagrai I, Ravelonandro M, Scorza R, Gaboreanu I, Ferenz B, Popescu O, Lumini A, Zagrai L, Maxim A (2007) Serological and molecular variability of *Plum pox virus* in transgenic and conventional plums. Acta Hortic 738:708–710

Zagrai I, Ravelonandro M, Scorza R, Mnoiu N, Zagrai L (2008a) Field release of transgenic plums in Romania. Bulletin of university of agricultural sciences and veterinary medicine Cluj-Napoca. Anim Sci Biotechnol 65:358–365 ISSN 1843–5262

Zagrai I, Capote N, Ravelonandro M, Cambra M, Zagrai L, Scorza R (2008b) *Plum pox virus* silencing of C5 transgenic plums is stable under challenge inoculation with heterologous viruses. J Plant Pathol 90:63–71

Zagrai I, Zagrai L, Ravelonandro M, Gaboreanu I, Pamfil D, Ferencz B, Popescu O, Scorza R, Capote N (2008c) Environmental impact assessment of transgenic plums on the diversity of *Plum pox virus* populations. Acta Hortic 781:309–318

Zagrai I, Ravelonandro M, Gaboreanu I, Ferencz B, Scorza R, Zagrai L, Kelemen B, Pamfil D, Popescu O (2011) Transgenic plums expressing the *plum pox virus* (PPV) coat protein gene do not assist the development of PPV recombinants under field conditions. J Plant Pathol 93:159–165

Zhang H, Harry DE, Ma C, Yucerr C, Hsu C-Y, Vikram V, Shevchenko O, Etherington E, Strauss SH (2010) Precocious flowering in trees: the *Flowering Locus T* gene as a ressearch and breeding tool in *Populus*. J Exp Bot 61:2549–2560

Zhebentyayeva TN, Reighard G, Lalli D, Gorina VM, Krška B, Abbott AG (2008) Origin of resistance to *plum pox virus* in apricot: what new AFLP and targeted SSR analyses tell. Tree Genet Genomes 4:403–417