Application for Determination of Non-regulatory Status for C5 ('HoneySweet') Plum (*Prunus domestica* L.) Resistant to *Plum Pox* Virus

Identifier: ARS-PLMC5-6

No CBI

Unfavorable information: NONE

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Executive Summary

Plum pox virus (PPV) is the causal agent of Sharka disease, the most serious virus disease of stone fruits which include peach, nectarine, plum, apricot and cherries. PPV is spread from tree to tree by aphids and through infected budwood used for grafting which is the normal method of propagating stone fruit trees. Symptoms of plum pox infection include leaf and fruit yellowing, fruit deformation, premature fruit drop and, when in the presence of other *Prunus* viruses, tree decline. Originally reported from Bulgaria, plum pox virus has spread throughout Europe where it has destroyed well over 100 million stone fruit trees. In the past decade, it has spread from the European continent to India, Egypt, Lebanon, the Azores, Chile, and most recently, the state of Pennsylvania in the U.S., Canada, Argentina and China. In the U.S. PPV is classified as an invasive species. An "invasive species" is defined as a species that is non-native (or alien) to the ecosystem under consideration *and* whose introduction causes or is likely to cause economic or environmental harm or harm to human health.

Sharka prevention is based on the use of certified virus-free plant material for new orchards, quarantine measures, and eradication of sources of infection. Once an orchard or growing area is infected, the only control option is tree eradication. In Pennsylvania alone, since the first report of sharka in 1999, 1600 acres of stone fruit trees have been destroyed at a cost of over $40 million.

PPV is not limited to commercial stone fruit trees. Over 40 *Prunus* species are now known to be susceptible to this virus. These include species native to North American woodlands including wild black cherry, a valuable lumber species, wild red cherry, sand cherry, choke cherry, big-tree plum, beach plum, chickasaw plum, American plum, and popular flowering *Prunus* ornamentals such as flowering cherry and dwarf flowering almond.

Few highly resistant stone fruit or ornamental *Prunus* varieties have been developed through conventional breeding. The rarity of confirmed sources of PPV resistance in *Prunus* and the difficulty in producing highly resistant varieties through conventional plant breeding make genetic engineering for resistance an attractive complementary approach to producing PPV resistant varieties. Genetic engineering using the plum pox virus coat protein gene produced a number of genetically modified (GM) plum clones including one clone, C5, that was found to be highly resistant to Sharka. The resistance of this clone is based on the mechanism of post-transcriptional gene silencing which has been found to be a natural mechanism in plants, including plum, for combating viruses. An interesting feature of this resistance mechanism is that no transgenic PPV coat protein is produced in C5.

The PPV resistance of C5 has proven to be highly stable in greenhouse and field tests. These field tests have been carried out for over eight years in three European countries where the virus is present (Poland, Romania, and Spain). No C5 trees in any of these field tests have become infected through natural aphid inoculation. Graft inoculated trees using infected budwood or trees growing on infected susceptible rootstocks showed few symptoms and when present symptoms were very mild. C5 trees are productive. The fruit are of high quality, flavorful, sweet, of large size, firm, and attractive. Research using C5 as a parent in producing new plum seedlings has

shown that the seedlings carrying the transgene from the C5 parent are also resistant to PPV.

C5 was selected for application for determination of non-regulatory status based on 1) the high level of resistance of C5 to *Plum pox virus*; 2) the stability and durability of resistance in the field under varying environmental conditions, including Mediterranean (Spain) and Continental (Poland and Romania) climates, and under pressure of different virus strains and mixtures of strains, and vector species; 3) the absence of PPV coat protein production; 4) the excellent quality of the fruit produced by C5; and 5) the ability of C5 to transmit the transgene insert and resistance to progeny in a Mendelian fashion as a dominant trait.

C5 can be a used as a commercial variety. It can also be used as a parent for breeding new resistant varieties through traditional plant breeding methods. C5 offers a potentially important tool for the protection of the *P. domestica* plum industry in the U.S. from sharka disease. This need clearly exists. In a recent issue of ***California Agriculture*** Gianessi (2004), referring to C5, wrote "If plum pox virus reaches California, the transgenic plum could help prevent losses to the state's multibillion dollar stone-fruit industry". Gianessi et al. (2002), writing for the National Center for Food and Agricultural Policy wrote "that a transgenic viral resistant stone fruit cultivar could be planted universally in Pennsylvania and would prevent the complete loss of production in the state (60 million pounds with a value of $17 million)."

Plum pox virus is currently the subject of a strict quarantine and eradication program in the U.S. (Pennsylvania). If PPV is eradicated there will not be a pressing need for the immediate deployment of C5 in commercial plantings. But the U.S. will always be vulnerable to this virus. A high level of genetic resistance as has been developed in C5, which can be rapidly deployed, is a critical component in a strategy to defend the U.S. against this important disease.

Nomenclature: Throughout this document the subject plum is referred to as "C5", which is the original designation of the selected clone. C5 has been patented under the name 'HoneySweet' US PP15,154 P2. To allow reviewers to better search the literature that has developed for this plum line in our laboratory and in other programs, the C5 designation has been used throughout this application.

Additional plum clones referenced in this application include:
C2 – a transgenic clone derived from a seedling of 'Bluebyrd' plum that carries several copies of the PPV-CP and marker transgenes. It produces a moderate amount of PPV-CP and is susceptible to PPV (Scorza et al., 1994; Hily et al, 2004).
C3 – a transgenic clone derived from a seedling of 'Bluebyrd' plum that carries several copies of the PPV-CP and marker transgenes. It produces a moderate amount of PPV-CP and is susceptible to PPV (Scorza et al., 1994; Hily et al, 2004).
C4 – a transgenic clone derived from a seedling of 'Bluebyrd' plum that carries approximately 3 copies of the PPV-CP and marker transgenes. C4 produces a high level of PPV-CP mRNA and coat protein. It is a clone that has shown some resistance to PPV in field tests when exposed to aphid-vectored infection, but resistance has been shown to break down during the course of field testing (Scorza et al., 1994; Hily et al, 2004).
C6 – a transgenic clone derived from a seedling of 'Bluebyrd' plum that carries only a partial copy of the transgene insert. The *uidA* (GUS) and part of the PPV-CP gene are deleted. No PPV-CP mRNA or protein is detected. C-6 is susceptible to PPV (Scorza et al., 1994; Hily et al, 2004).
C3-2 – A transgenic plum originally labeled as transgenic clone C3 but following DNA blotting it was found to be a transgenic C2 plant.
'Bluebyrd' – A seedling originally designated as B69158 that was obtained from open-pollination of plum selection NY H4. NY H4 originated from the New York Agricultural Experiment Station, Geneva, NY. 'Bluebyrd' was the female parent of the C-series of transgenic plums listed above including C5. The male, or pollen parent of these transgenic clones is unknown. 'Bluebyrd' is a self-sterile, productive, high quality *Prunus domestica* plum that was publicly released on March 20, 1998 (Scorza and Fogle, 1999). This clone is non-transgenic.
B70146 - A seedling from open-pollination of plum selection NY 215. NY 215 originated from the New York Agricultural Experiment Station, Geneva, NY. This clone is non-transgenic.

I. Rationale for Development of C5 plum

Plum pox virus and "Sharka" Disease. Plum pox virus (PPV: family *Potyviridae*, genus *Potyvirus*) is the causal agent of sharka disease, the most serious virus disease of stone fruits which include peach, nectarine, plum, apricot and cherries (Dunez and Sutic, 1988). PPV is spread from tree to tree by aphids (Kunze and Krczal, 1971) and through the use of infected budwood. Symptoms of plum pox infection (Sharka Disease) include leaf and fruit chlorosis, fruit deformation, premature fruit drop and, in the presence of other *Prunus* viruses, tree decline. Originally reported from Bulgaria (Atanassov, 1932), plum pox virus has spread throughout Europe (Al Rwahnih et al., 2001) where it has destroyed well over 100 million stone fruit trees. In the past decade, it has spread from the European continent to India, Egypt, Lebanon, the Azores, Chile (Rosales et al., 1998), and most recently, the state of Pennsylvania in the U.S. (Levy et al., 2000), Canada (Thompson et al., 2001), Argentina (http://archives.foodsafetynetwork.ca/agnet/2005/5-2005/agnet_may_28.htm) and China (Navratil et al., 2005).

In the U.S. PPV is classified as an invasive species. An "invasive species" is defined as a species that is non-native (or alien) to the ecosystem under consideration *and* whose introduction causes or is likely to cause economic or environmental harm or harm to human health.

Sharka prevention is based on the use of certified plant material for new orchards, quarantine measures and eradication of sources of infection. Once an orchard or growing area is infected, the only control option is tree eradication. In Pennsylvania alone, since the first report of sharka in 1999, 1600 acres of stone fruit trees have been destroyed at a cost of over $40 million.

PPV is not limited to commercial stone fruit hosts. Over 40 *Prunus* species are now known to be susceptible to this virus (Damsteegt et al., 2004). These include species native to North American woodlands including wild black cherry (*P. serotina*), a valuable lumber species, wild red cherry (*P. pensylvanica*), sand cherry (*P. besseyi*), choke cherry (*P. virginiana*), big-tree plum (*P. mexicana*), beach plum (*P. maritima*), chickasaw plum (*P. angustifolia*), American plum (*P. americana*), and popular flowering *Prunus* ornamentals such as flowering cherry and dwarf flowering almond.

Breeding for resistance. Prunus (peach, plum, apricot, cherry and almond) cultivars have been described as 'susceptible', 'tolerant', 'resistant' or 'immune' to PPV, but contradictory evaluations have made the interpretation of these ratings problematic (Kegler et al., 1998). Few highly resistant cultivars have been developed by breeding or grower selection. The development of genetic markers related to resistance genes in apricot is being pursued by several programs (Abernathy et al., 2004; Dondini et al., 2004). When it has been identified, resistance appears to be multigenic (Neumüller et al., 2005).

The lack of readily identifiable and reliable sources of PPV resistance in *Prunus* and the difficulty in producing highly resistant cultivars through conventional plant breeding, suggested the utility of pathogen-derived resistance (PDR) (Sanford and Johnston, 1985) for providing PPV resistance. This technology was first

demonstrated by expression of the coat protein (CP) gene of *Tobacco mosaic tobamovirus* (TMV) in transgenic tobacco plants (Powell-Abel et al., 1986). Since this first report, expression of CP gene sequences in transgenic plants has become a well-established and effective strategy to protect plants from potyvirus infection (Fitch et al., 1992; Lindbo and Dougherty, 1992; Smith et al., 1995; Jan et al., 1999; Sonoda et al., 1999). Ravelonandro et al. (1993) demonstrated pathogen-derived PPV resistance in the herbaceous model *Nicotiana benthamiana* using the coat protein (CP) gene of PPV. Based on this work, plum (*Prunus domestica* L.) was transformed with the PPV-CP gene (Scorza et al., 1994). From this work, a number of transgenic clones were produced including one clone, C5 that was found to be highly resistant to PPV (Scorza et al., 1994; Ravelonandro et al., 1997; Hily et al., 2004).

The PPV resistance of C5 has proven to be highly stable in greenhouse and field tests. These field tests have been carried out for seven to eight years in three European countries where the virus is present (Poland, Romania, and Spain) (Hily et al., 2004). No C5 trees in any of these field tests have become infected through natural aphid inoculation. Graft inoculated trees or trees growing on PPV-infected rootstocks, show few symptoms of a mild nature and have a very low virus titre. Movement of the virus from the graft union is severely limited (Malinowski et al., 2006). When used as a parent in hybridizations it was shown that C5 transferred PPV resistance to its progeny thus making it a useful parent in PPV resistance breeding programs (Scorza et al., 1998; Ravelonandro et al., 2001).

Interestingly, C5 while being the most resistant clone that we tested was also the clone that produced a low level of PPV-CP messenger RNA and no detectable PPV coat protein. Over the last several years many research laboratories have begun to unravel the mechanism underlying this resistance phenotype. These studies have provided evidence that PDR in these cases is based on RNA silencing, termed post-transcriptional gene silencing (PTGS) in plants and RNA interference in animal systems (Kooter at al., 1999; Matzke et al., 2001; Vaucheret et al., 2001; Waterhouse et al., 2001, Hannon 2002). RNA silencing leads to the degradation of homologous mRNAs. In plants, RNA silencing not only participates in the natural regulation of endogenous gene expression in developmental processes, but also serves as a component of adaptative protection against mobile genetic elements, such as transposons and viruses (Voinnet, 2005). A key component of the pathway is known as short interfering RNA (siRNA) (Hamilton and Baulcombe, 1999) that derive from double stranded (ds) RNA, which seem to play a central role in triggering sequence specific RNA degradation. These siRNAs, corresponding to both sense and antisense strands, guide a multi-subunit ribonuclease, the RNA-induced silencing complex (RISC), and ensure that it specifically degrades RNAs that share sequence similarity with the dsRNA. The expression of dsRNA triggers the PTGS pathway with the degradation of the dsRNA by a dsRNA specific RNase III enzyme termed DICER (Bernstein et al, 2001). This process produces siRNA of 21 to 25 nt which are diagnostic for PTGS (Hamilton and Baulcombe, 1999). As our data indicate C5 presents all of the hallmarks of PTGS including siRNA. We show that C5 contains both the long and short species of siRNA. The production of the short species (~22 nt) occurs in PPV infected non-transgenic plums indicating that PTGS is a natural reaction in plum to virus infection (Hily et al., 2005).

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C5 can be a useful cultivar or breeding parent in countries where Sharka Disease is an emerging threat or where the disease is endemic. C5 offers a potentially important tool for the protection of the *P. domestica* plum industry in the U.S. from sharka disease. This need clearly exists. In a recent issue of ***California Agriculture*** Gianessi (2004), referring to C5, wrote "If plum pox virus reaches California, the transgenic plum could help prevent losses to the state's multibillion dollar stone-fruit industry" (Appendix A). Gianessi et al. (2002), writing for the National Center for Food and Agricultural Policy wrote "that a transgenic viral resistant stone fruit cultivar could be planted universally in Pennsylvania and would prevent the complete loss of production in the state (60 million pounds with a value of $17 million)" (Appendix B).

II. The Genus *Prunus*

Taxonomy (from OECD, 2002). In the past, different approaches have been used to present the phylogeny of the subfamily of *Prunoideae* belonging to the family of *Rosaceae*. There were two main contrasting conceptions, i.e. all stone fruits belong to the genus *Prunus,* or the genus *Prunus* contains only plums and prunes. The *OECD Consensus Document on the Biology of Prunus sp. (Stone Fruits)* (2002) has presented the classification according to Strasburger et al. (1991). The seven subgenera in *Prunus* are determined basically by how the leaves are rolled up in the bud, whether the flowers are organised in cymes or in racemes and by morphological characteristics of the generative organs, i.e. the size and colour of flowers, fruit, stone and seed traits.

- AMYGDALUS (almonds): ***P. amygdalus***, *P. bucharica, P. fenzliana, P. kuramica, P. nana, P. orientalis, P. webbii*
- PERSICA (peaches): *P. davidiana, P. ferganensis, P. kansuensis, P. mira,* ***P. persica***
- ARMENIACA (apricots): *P. ansu,* ***P. armeniaca***, *P. brigantiaca, P. x dasycarpa, P. holosericea, P. mandshurica, P. mume, P. sibirica*
- PRUNUS (plums and prunes): *P. cerasifera, P. divaricata,* ***P. domestica***, *P. insititia, P. italica, P. spinosa, P. syriaca,* ***P. salicina***, *P. simonii, P. ussuriensis, P. americana, P. angustifolia, P. hortulana, P. maritima, P. mexicana, P. munsoniana, P. nigra, P. rivularis, P. subcordata*
- CERASUS (sweet and sour cherries): ***P. avium, P. cerasus***, *P. fruticosa, P. japonica, P. maackii, P. mahaleb, P. pseudocerasus, P. pumila, P. serrulata, P. tomentosa*

- PADUS (bird cherries) *P. padus, P. serotina*
- LAUROCERASUS (bay-cherries)

The species in bold (*P. amygdalus, P. persica, P. armeniaca, P. domestica, P. avium, P. cerasus* and *P. salicina)* are the most widely grown species with horticultural interest.

Chromosome Numbers. In *Prunus*, the basic number in vegetative cells is eight chromosomes. Polyploidy, due to interspecific hybridization, took place during the phylogeny of the genus and is responsible for self-sterility and intersterility.

The following number of chromosomes and degrees of ploidy has been reported:

Sub-genus	**Species**	**Chromosome number**	**Reference**
Amygdalus	*P. amygdalus*	2n=16 (diploid)	Darlington et al., 1945
	P. bucharica	2n=16 (diploid)	
	P. fenzliana	2n=16 (diploid)	Darlington et al., 1945
	P. kuramica	2n=16 (diploid)	
	P. orientalis	2n=16 (diploid)	
	P. tenella	2n=16 (diploid)	Darlington et al., 1945
Persica	*P. davidiana*	2n = 16 (diploid)	Missouri Botanical Garden, 1990
	P. ferganensis	2n = 16 (diploid)	Missouri Botanical Garden, 1991
	P. kansuensis	2n = 16 (diploid)	Missouri Botanical Garden, 1990
	P. mira	2n = 16 (diploid)	
	P. persica	2n = 16 (diploid)	Darlington et al., 1945 Bennett and Leitch, 1995

Armeniaca	*P. ansu*	2n = 16 (diploid)	
	P. armeniaca	2n = 16 (diploid)	Darlington et al., 1945 Bennett and Leitch, 1995
	P. brigantiaca	2n = 16 (diploid)	
	P. mandshurica	2n = 16 (diploid)	
	P. x dasycarpa	2n = 16 (diploid)	
	P. holosericea	2n = 16 (diploid)	
	P. mume [1/]	2n = 16 (diploid), 24	Darlington et al., 1945
	P. sibirica	2n = 16 (diploid)	

[1/] These species have been reported in differing ploidy levels.

All apricot species are diploids with eight pairs of chromosomes (2n=16). No difficulties have been reported in intercrossing *P. armeniaca, P. siberica, P. mandshurica* and *P. mume*, although not all combinations have been attempted. *P. x dasycarpa* Ehrh., the black or purple apricot, is a naturally occurring hybrid of *P. cerasifera* Ehrh. and *P. armeniaca,* and is found as isolated trees, where the distribution of the two species overlaps (Mehlenbacher et al., 1991). *P. x dasycarpa* has been backcrossed to both *P. cerasifera* and *P. armeniaca*; crosses to the plum parent are generally easier.

Sub-genus	Species	Chromosome number	Reference
Prunus	*P. angustifolia*	2n=16 (diploid)	Janick and Moore, 1975
	P. cerasifera	2n=16 (diploid), 24	Janick and Moore, 1975
	P. domestica	2n=48 (hexaploid)	Darlington et al., 1945 Bennett and Leitch, 1995
	P. hortulana [1/]	2n=16 (diploid) 2n=48 (hexaploid), 24	Janick and Moore, 1975 Darlington, 1945, Tischler, 1950
	P. italica	2n=48 (hexaploid)	
	P. maritime	2n=16 (diploid)	Darlington et al., 1945

[1/] These species have been reported in differing ploidy levels.

	P. mexicana	2n=16 (diploid)	Janick and Moore, 1975
	P. munsoniana	2n=16 (diploid)	Janick and Moore, 1975
	P. nigra	2n=16 (diploid)	Darlington et al., 1945
	P. rivularis	2n=16 (diploid)	Janick and Moore, 1975
	P. salicina	2n=16 (diploid)	Darlington et al., 1945
	P. simonii	2n=16 (diploid)	Janick and Moore, 1975
	P. spinosa [1/]	2n=32 (tetraploid), and natural hybrids with 16, 24, 40, 48	Darlington et al., 1945, Janick and Moore, 1975
	P. subcordata	2n=16 (diploid)	Janick and Moore, 1975
	P. salicina	2n=16 (diploid)	Darlington et al., 1945, Jannick and Moore, 1975
	P. syriaca	2n=16 (diploid)	
	P. ussuriensis	2n=16 (diploid)	

[1/] These species have been reported in differing ploidy levels.

The most important commercial species of plums are generally classified in two groups, the European plums (*Prunus domestica* L.) and related forms with hexaploid chromosome number (2n=6x=48) and the Japanese plums (*Prunus salicina*) and their hybrids with diploid chromosome number.

Sub-genus	**Species**	**Chromosome number**	**Reference**
Cerasus	*P. avium* [1/]	2n=16 (diploid), 24, 32	Darlington et al., 1945 Bennett and Leitch, 1995
	P. besseyi	2n=16 (diploid)	
	P. cerasus	2n=32 (tetraploid)	Missouri Botanical Garden, 1985 Bennett and Leitch, 1995

	P. fruticosa	2n=32 (tetraploid)	
	P. mahaleb	2n=16 (diploid)	Darlington et al., 1945
	P. pumila	2n=16 (diploid)	Darlington et al., 1945
	P. serrulata[1/]	2n=16 (diploid), 24	Darlington et al., 1945
	P. subhirtella	2n=16 (diploid)	Darlington et al., 1945 Bennett and Leitch, 1995
	P. tomentosa	2n=16 (diploid)	Darlington et al., 1945

[1/] These species have been reported in differing ploidy levels.

Among cherries, the sweet (*P. avium*) and sour (*P. cerasus*) cherry, flowering ornamental cherry species, and a few others used as rootstocks for cherries are important. P.fr*uticosa*, the ground cherry, is considered the probable parent of both *P. avium* and *P. cerasus*, sweet and sour cherry respectively (Fogle, 1975). The chromosome number of *P. cerasus* is 32 (Crane and Lawrence, 1952). As 8 is the base number of the genus *Prunus* and following De Candolle´s hypothesis (Coutanceau 1953), it seems that sour cherry is a tetraploid originating from an unreduced *P. avium* (2n = 16) gametophyte, thus by chromosome doubling.

Sub-genus	Species	Chromosome number	Reference
Padus	*P. padus*	2n=16 (diploid)	Tischler, 1950
	P. serotina	2n=32 (tetraploid)	Kumar and Subramanian, 1987 Bennett and Leitch, 1995

III. *Prunus domestica* (European or Prune Plum) as a Crop

Geographic origin and natural distribution of P. domestica. Plums are a diverse group of plants with many botanical species that have been cultivated for the last 3000 years. The most important species of the sub-genus *Prunus* are generally classified into three groups: the European, the Asian and the American plums (**Table 1**). Plums may have been the first species among all the fruits to attract human interest. Six of the most important species of plums, *P. domestica, P. italica, P.*

syriaca, P. salicina, P. simonii and P. americana, are not known in the wild and presumably were selected and cultivated very early by humans. *Prunus domestica* seems to have originated in Southern Europe or Western Asia around the Caucasus Mountains and the Caspian Sea (Cullinan, 1937). However, it is also widespread in the Balkans and Mediterranean countries.

Table 1. The classification of plums is divided into geographic groups:

European group	*P. spinosa L.*	Europe, Asia Minor and North Africa
	P. cerasifera Ehrh. (and *P. divaricata* Ledeb.)	With some eco-geographical subspecies in Balkan, Asia Minor, Caucasian region and Central Asia
	P. insititia L.	In Central Europe, Balkan, Western Asia
	P. domestica L.	Native in Western Asia
	P. italica (Borkh.) em. Kárpáti	Hybrid between *P. domestica* and *P. insititia* with convarietas (*pomariarum, claudiana, ovoidea and mamillaris)*
	P. syriaca (Borkh.) em. Kárpáti	Hybrid between *P. cerasifera* and *P. domestica*
Asian group	*P. salicina*	Native in the Basin of Yangtze River
	P. simonii	No wild form, only cultivated
	P. ussuriensis	Along Ussuri River
American group	*P. subcordata*	Native of California and Oregon
	P. mexicana	South-western Kentucky to Western Tennessee to Oklahoma and Mexico
	P. rivularis	Native in Texas
	P. maritima	From Brunswick to Virginia
	P. americana	From Massachusetts to Georgia to near the Gulf of Mexico and to the west
	P. nigra	From New Brunswick to Northern Ohio
	P. angustifolia	From Delaware to Florida and Texas
	P. hortulana	Native in Central Kentucky and Tennessee, to Iowa and Oklahoma
	P. munsoniana	From Kentucky to Kansas and Texas

World Production. World production of all plums in 2003 was 10,109,515 Mt with *P. domestica* accounting for a large part of that production. The ten highest producing countries are listed in **Table 2**.

Table 2. Major plum producing countries (all plum species):

Plums Production (Mt)	Year 2003
China	4,234,419
Romania	909,648
United States of America	725,290
Serbia and Montenegro	577,431
Germany	478,730
France	246,700
Chile	240,000
Turkey	205,000
Spain	196,400
Russian Federation	156,000

U.S. Production. California is the leading producer of dried (*P. domestica*) plums in the U.S. and accounts for more than 95% of the value of U.S. production. In 2002, 171,000 tons of dried plums were produced in California on over 70,000 acres with a farm gate value of $132.2 million. The U.S. is a net exporter of dried plums with all exports originating in California. In 2002, the export value was $133.5 million. California produces approximately 99% of the U.S. supply and 70% of the world supply.

IV. Genetics of *P. domestica*

Origin of P. domestica and pollination biology. P. domestica is believed to have arisen as a natural alloploid between *Prunus cerasifera* (diploid) and *P. spinosa* (tetraploid) (Crane and Lawrence, 1952). Many *P. domestica* cultivars are self-incompatible and may be cross-incompatible or cross-compatible depending on the compatibility genotype. Incompatibility may be due to gametic or sporophytic incompatibility. Both systems are complex, based on multiple alleles originating from different progenitors making up the hexaploid *P. domestica* genome (Botu et al., 2002). *P. domestica* pollen, like pollen of *Prunus* species in general, is insect transferred and is not normally windborne (Weinberger, 1975). Most cultivated *Prunus* species, including peach, nectarine, and Japanese plum and wild species, are diploid (Section II *Chromosome numbers*) and do not naturally hybridize with hexaploid *P. domestica*. Peach (*P. persica*) is a species that most readily hybridizes with other *Prunus* species. It has been reported to produce sterile hybrids with *P. domestica* (OECD, 2002). Successful crosses between apricot and *P. domestica, and* between *P. domestica* and other species in the apricot (*Armeniaca*) and plum (*Prunus*) groups has been indicated in the OECD 2002 ***Consensus Document on the Biology of Prunus sp. (Stone Fruits)***, but citations of such work were not included in this report, and the viability and fertility of these purported hybrids was not discussed.

Okie and Weinberger (1996) also refer to studies of interspecific hybridization with *P. domestica* and suggest that the yields of hybrids that are produced are low. Botu et al. (2002) reported successful hybridization between *P. domestica* and *P. institia* (6x), *P. spinosa* (4x), *P. besseyi* (2x), and [(*P. besseyi x P. americana*) (2x)]. Most hybrids were sterile. The difficulties of interspecific hybridization within the genus *Prunus* have been discussed by Layne and Sherman (1986).

Introgression into Wild Relatives (from OECD, 2002). The introgression between cultivated and wild species is scarcely documented. There is no doubt concerning the physical possibility. Escapes of cultivated varieties are frequently found in woods, pastures, abandoned orchards, rural, suburban and marginal areas. Intercrosses with really wild populations have very little chance, as blackthorn, hedge cherry and dwarf almond (*P. tenella*) are extremely different in morphology, as well as in adaptation, i.e. eventual hybrids could only survive in a much protected environment. Cherries may have more chances as far as introgression into the wild populations is concerned. It is worthwhile to consider the escapes of varieties and species introduced as rootstocks to nurseries and grown out from the roots and stumps of destroyed grafts in abandoned orchards. That is how a high diversity of cherry plums (*P. cerasifera*) has been naturalized recently in Europe. As a result, the cherry plum has become much more tolerant than the European plum and apricot to the destructive effect of PPV. Escaped rootstock varieties and spontaneous hybrids of ancient, as well as, recently introduced varieties are a general phenomena found in neglected orchards, and escapes of no immediate relation to fruits growing in the area are found. For example, *P. serotina, P. mahaleb, P. padus,* bitter almonds.

In Central Europe, the possibility of introgression is more limited to the Near East, Caucasus, Iran, Central Asia and the Chinese subcontinent, where a huge wealth of intermediate and semi-cultivated forms reside.

In the U.S., particularly in the mid-Atlantic states, the potential for gene transfer to native of naturalized *Prunus* species is quite limited based on ploidy differences (**Table 3**), on the lack of documented natural outcrossing, and on the limited success in the production of *P. domestica* interspecific hybrids through controlled breeding.

Table 3. Ploidy of Prunus species, native, naturalized and commercial in the states of Pennsylvania (Rhodes and Klein, 1993) and Maryland (Reveal, 2002).

Species	Ploidy[1]
americana (American plum)	2n
armeniaca (apricot)	2n
avium (sweet cherry)	2n
cerasifera (myrobalan plum)	2n
cerasus (sour cherry)	4n
domestica (European plum)[2]	6n
hortulana (wild-goose plum)	2n
insititia (damson plum)[2]	6n
mahaleb (mahaleb cherry)	2n
maritima (beach plum)	2n
padus (bird cherry)	4n

pensylvanica (pin cherry)[3,4]	2n, 4n
pumila (sand cherry)	2n
serotina (wild black cherry)	4n
spinosa (blackthorn)	4n
triloba (flowering almond)	8n
virginiana (chokecherry)	4n
persica (peach)	2n

[1] Darlington and Wylie, 1945
[2] Potential for fertile hybrids with *P. domestica*
[3] Great Plains Flora Association
[4] Gleason and Cronquist, 1991

Weediness of P. domestica. Most *P. domestica* clones produce root suckers or tillers. This tendency varies with genotype. Some successful cultivars, i.e. Pozegaca in NE-Hungary, have been maintained by tillers since ancient times. No references to *P. domestica* as a weedy species have been found. Further, none of the *Prunus* species that may be sexually compatible with *P. domestica* are classified weedy species.

Potential for gene escape in P. domestica. The greatest potential for gene escape is into other *P. domestica* clones. Our results to date from field trials indicates that the PPV-CP, NPTII, and GUS transgenes can be passed naturally to compatible *P. domestica* clones (unpublished). Gene escape to other *Prunus* species seems less likely since only few species have been shown to be compatible with *P. domestica* through controlled hybridization, and of the hybrids produced, few are fertile [see sections IV *Genetics of P. domestica*, IV *Introgression into Wild Relatives*, and XII *Gene flow from transgenic P. domestica plum trees*].

V. Description of the Transformation System

Plant material. The transformation system was described in Mante et al. (1991) and in Scorza et al. (1994). Briefly, seeds of the plum cultivar 'Bluebyrd' were collected and used fresh, when seed were filled but fruit were still immature, fresh from mature fruit, or after storage at 4 °C for up to 10 months. Seeds were removed from the fruit prior to cold storage. Stored seeds were disinfected in a 0.5% sodium hypochlorite solution with 0.005% Tween 20 for 12-15 min., washed 3 times with sterile deionized water and soaked in sterile deionized water overnight at room temperature. All further manipulations were done under sterile conditions. The seed coat was removed and the cotyledons were slit open. The embryonic axis was removed and cut with a scalpel into three segments: epicotyl, hypocotyl, and radicle. The hypocotyl segment was sliced in thirds with each slice 0.5-1 mm thick. These slices were used for transformation.

Agrobacterium strain and plasmid description. Construction and use of the binary plasmid pGA482GG containing the NPTII and GUS marker genes has been described (Fitch et al, 1990; Ling et al. 1991). The PPV-CP gene cassette with 35S promoter

from the plasmid pBIPCP (Ravelonandro et al. 1992) was subcloned into the binary plasmid pGA482GG after digestion with *Hind*III. The resulting plasmid was designated pGA482GG/PPV-CP-33 and was used to electrotransform *A. tumefaciens* strains C58/Z707 **Fig. 1**. *A. tumefaciens* were grown overnight at 28 °C in 10 ml Luria broth with kanamycin (kan) at 50 µg/ml and gentamicin at 50 µg/ml as selective agents. These cultures were then centrifuged (4000 x g, 10 min), resuspended in 10 ml of bacterial resuspension medium (BRM) consisting of Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962) with 2% sucrose, 100 µM acetosyringone and 1 mM betaine phosphate, and shaken for 6 h at 20 °C before use.

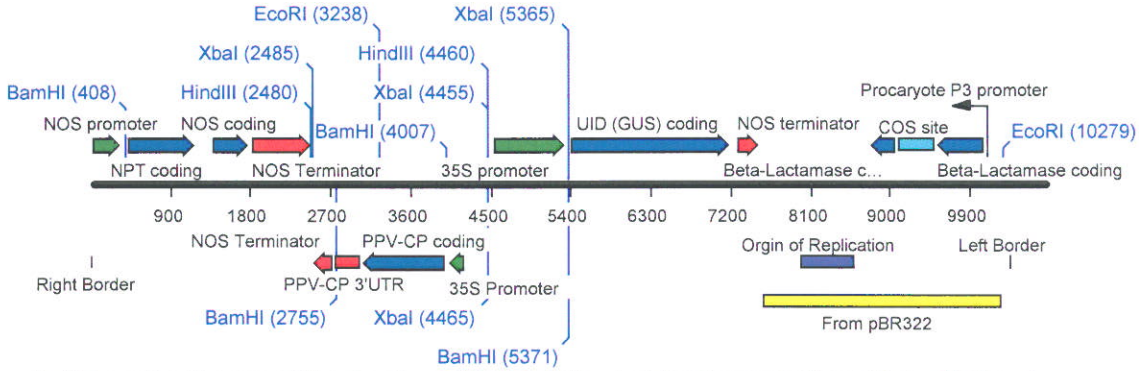


Figure 1. Schematic diagram of the structure of the cassette used for plum transformation with the *plum pox virus* (PPV) coat protein (CP) gene. Blue indicates coding regions, green = promoters, red = terminators. The portion of the insert derived as a cloning artifact from plasmid pBR322 is indicated with a light green bar with the origin of replication indicated in red. The *Bam*HI-isolated PPV-CP fragment is 1.2 kb.

Transformation and regeneration. Shoot regeneration medium (SRM) consisted of MS salts and vitamins plus indolebutyric acid (IBA) at 0.25 µM and thidiazuron (TDZ) at 7.5 µM. Co-cultivation was carried out by immersing hypocotyl slices in the resuspended *A. tumefaciens* for 10-20 min, blotting them briefly and placing them on cocultivation medium (SRM containing 100 µM acetosyringone). Following a 48 h cocultivation period the explants were washed 3 times in half strength liquid MS medium containing 500 µg/ml carbenicillin and 200µg/ml cefotaxime. Explants were then placed on agar-solidified SRM containing carbenicillin and cefotaxime in the same concentrations as the wash and cultured for 10-14 days. Following this culture period, explants were transferred to SRM with carbenicillin and cefotaxime as above with 75 µg/ml kan. Only one regenerated shoot was selected from each hypocotyl slice. Regenerated shoots were subcultured onto fresh selective medium (SRM with antibiotics) every 3-4 weeks until they were confirmed transgenic.

Selection of putative transformants. Shoots that grew in the presence of 75 µg/ml kan were assayed for GUS activity, Leaf tissue cut from these shoots was incubated for 16 to 24 hours in an X-Glu solution (Jefferson 1987) at 37 °C and blue staining of the tissue observed. Confirmation of transformation was through PCR for the PPV-CP and marker genes (NPTII and GUS).

VI. Donor Genes and Regulatory Sequences

Components of the plasmid used in the development of transgenic *P. domestica* C5 are listed and described in **Table 4**.

Table 4. Components of the plasmid used in the development of transgenic *P. domestica* C5.

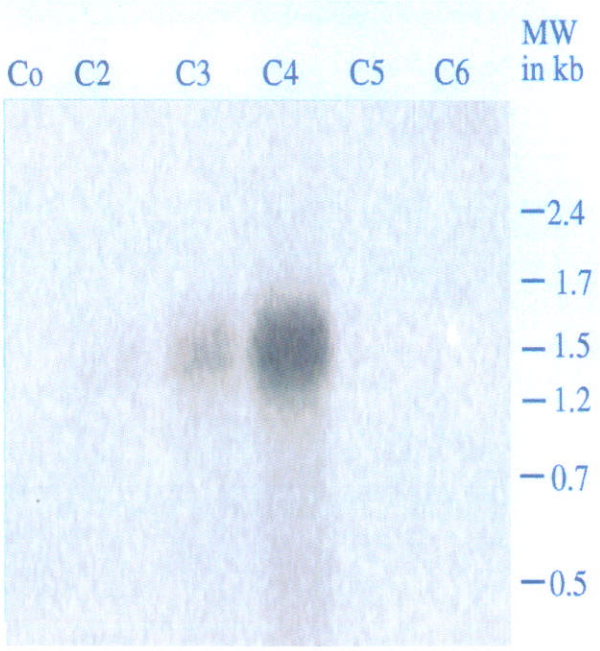
Genetic Element	Size (Kb)	Function, Source, Reference
RB	0.16	A restriction fragment from the pTiT37 plasmid containing the 24 bp nopaline-type T-DNA right border used to initiate the T-DNA transfer from *Agrobacterium tumefaciens* to the plant genome (Depicker et al., 1982)
nos 5'	0.25	Nopaline synthase promoter from A*grobacterium tumefaciens* (An, 1986; Bevan et al., 1983)
nptII	0.79	Neomycin phosphotransferase type II from Tn5 (Rothstein et al., 1981)
nos 3'	1.20	A 3' non-translated region of the nopaline synthase gene which functions to terminate transcription and direct poly adenylation of the nptII mRNAs (Depicker et al., 1982; Bevan et al., 1983)
nos 3'	0.25	A 3' non-translated region of the nopaline synthase gene which functions to terminate transcription and direct poly adenylation of the PPV-CP mRNAs (Depicker et al., 1982; Bevan et al., 1983)
PPV-CP	1.25	Insert containing the leader sequence of the coat protein mRNA of TMV and an ATG start codon fused in phase with the *plum pox* virus coat protein gene from PPV-D strain (Ravelonandro et al., 1992; Takamatsu et al., 1987)
CaMV (35 S)	0.45	The cauliflower mosaic virus (CaMV) promoter (Odell et al., 1985) to drive the expression of the PPV-CP gene.
CaMV (35)	0.85	The cauliflower mosaic virus (CaMV) promoter (Odell et al., 1985) to drive the expression of the GUS gene.
uidA (GUS)	1.81	β-glucuronidase gene (Jefferson, 1987), from *E. coli*
nos 3'	0.25	A 3' non-translated region of the nopaline synthase gene which functions to terminate transcription and direct poly adenylation of the GUS mRNAs (Depicker et al., 1982; Bevan et al., 1983)
Sequence from Cosmid MUA10 Originally from pBR322	**2.78**	**For details see below**
MUA10	0.40	cosmid sequence
Origin of replication	0.65	pBR322 sequence responsible for initiating plasmid replication.

MUA10	0.15	cosmid sequence
β lactamase	0.30	pBR322 gene for resistance to ampicillin
cos[1/]	0.45	a bacteriophage lambda cos site. The cos site results in efficient packaging of lambda DNA into virus particles
β lactamase	0.55	pBR322 gene for resistance to ampicillin
MUA10	0.20	cosmid sequence
LB	0.08	restriction fragment from the octopine Ti plasmid, pTil5955, containing the 24 bp TDNA left border used to terminate the transfer of the T-DNA from *Agrobacterium tumefaciens* to the plant genome (Barker et al., 1983)

[1/] The *cos* site interrupts the β-lactamase gene rendering it non-functional [see section VIII. Characterization of the DNA inserted into C5. *Interruption and inactivation of the β-lactamase (ampicillin resistance) gene.*]

VII. RNA Characterization and Expression.

RNA Blot Analysis. Total RNA was isolated from five transformed plants (C2 through C6) as well as the control plant ('Bluebyrd') to determine the relative amounts of PPV-CP-related RNA. **Fig. 2** demonstrates that plants C2 through C5 had an RNA transcript of approximately 1.4 kb. The transcript was not detected in the control. The amount of 1.4kb transcript detected varied with the four samples in that a 40 hour exposure was necessary to detect RNA in sample C5 while after five hours CP mRNA was detected in C2, C3 and C4.



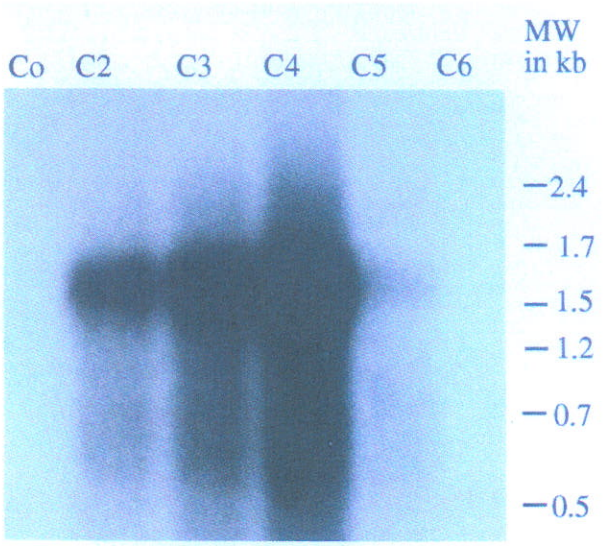


Figure 2. RNA blot of plum leaves from transgenic plum clones C2-C6 after 5 h exposure (top) and 40 h exposure (bottom). Co is the untransformed control. Total RNA (5 μg) was resolved on a hydroxy-methyl mercury gel, transferred electrophoretically to Nytran, stained with methylene blue to verify evenness of transfer and loading, prehybridized in Hybrisol at 42 °C and hybridized for 16 h with 32P-labeled-random primed PPV-CP fragment. The blots were washed three times with 0.1 X SSC at room temperature and then washed twice with 0.1X SSC at 65 °C for 30 minutes with a final wash of 0.1 X SSC at 52 °C and exposed to film with two intensifying screens at –80 °C. (A positive control was not included because the virus produces a single unprocessed RNA that includes CP and a number of additional viral RNA components including HC-Pro, Replicase, etc). Note the low level of transcript in clone C5 (Scorza et al., 1994).

Immunoblot Analysis. A protein product in the analyzed samples was immunoreactive with antiserum to PPV-CP and this product comigrated with purified PPV-CP **Fig. 3**. In the lane corresponding to the total soluble protein from the untransformed plant, no PPV-CP could be detected. The amount of CP in transgenic plums is different among the independent clones tested (**Fig. 3**). Clones C2, C3 and C4 had approximately 1.0 ng of detectable protein as determined by densitometer scanning while CP could not be detected in C5 and C6.

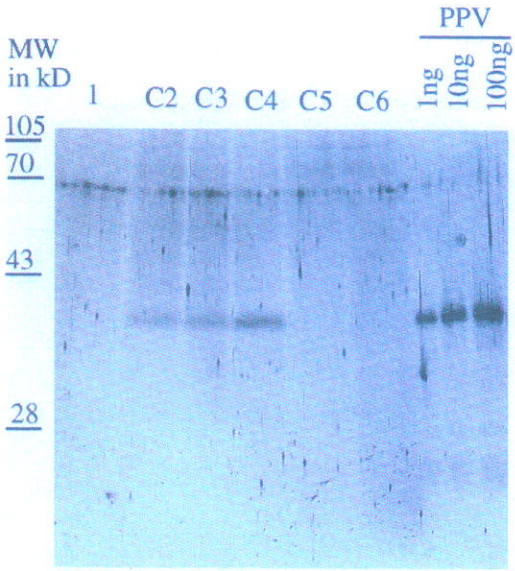


Figure 3. Immunoblot assay of transgenic plums C2 through C6. Blots (100 µg protein) were reacted overnight at 4 °C with 5 µl polyclonal antibodies against PPV-CP (Ravelonandro et al., 1992). Lane 1 is untransformed plum, MW represents protein standards resolved on the same gel and blotted. Ten or 100 ng of PPV were used to approximate the relative amounts of PPV-CP expressed in transgenic plants. Note the absence of detectable PPV-CP in clone C5 (Scorza et al., 1994).

Summary of PPV-CP RNA and Protein Analyses of C5. In summary, clone C5 had significantly lower amounts of RNA transcript in comparison to other transgenic lines and *no detectable PPV coat protein.*

MATERIALS AND METHODS FOR MOLECULAR CHARACTERIZATION OF TRANSGENIC CLONE

Polymerase chain reaction (PCR). PCR amplification was conducted on DNA isolated from leaves of putative transformed plum clones. Specific oligonucleotide primers at the 5' and 3' end of the PPV-CP gene were used to identify the presence of this gene in DNA isolated from different transgenic clones. The primers used for the PPV-CP gene were from the 3' region of the genome of PPV-D strain (Ravelonandro et al,. 1988) starting at bp 1212 (5') to bp 1190 (3'), ie. 5'-AAGCTGACGAAAGAGAGGACGAG'; 3'-primer, starting at bp 217 (5') to bp 240 ie. 5'-CTACACTCCCCTCACACCGAGGAA-3'. PCR reactions were run using the GeneAmp kit components (Perkin-Elmer, Norwalk, CT) and the following cycle parameters: 1 min at 94° C, 1.5 min at 65° C, and 2 min at 72° C. The first cycle used an additional 3 min melt at 95° C and the last 5 cycles had 4 min extension times at 72° C. After 35 amplification cycles the PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

RNA' and DNA extraction. RNA and DNA were extracted from young leaves of greenhouse--grown plants. Leaves were collected and frozen in liquid nitrogen, 0.3 g of leaf material was added to 20 ml of 65°C extraction buffer containing Proteinase K. The leaf tissue was phenol extracted, phenol-chloroform extracted, chloroform extracted, LiCI precipitated and ethanol precipitated. The RNA was quantitated at OD 260 and visualized on a gel to verify integrity.

DNA was extracted from fresh-frozen leaves by a modification of a protocol of Doyle and Doyle (1990) as previously described (Callahan et al, 1992). The leaf material was resuspended in a CTAB buffer, chloroform extracted, RNased,

phenol-chloroform extracted, ethanol precipitated and ethanol precipitated again with NH4-acetate. The DNA was visualized on a gel for integrity and amount using known standards.

Probe DNA. A fragment of PPV-CP gene was generated through a PCR reaction using the above primers with the following parameters: 1 min at 94 °C, 3 min at 55 °C and 2 min at 72 °C for 25 cycles, with the first cycle having a 5 min 95 °C start and the last cycle having a 15 min 72 °C end. The reaction was ethanol precipitated, eluted through an elutipD column per manufacturer's conditions (Schleicher and Schuell, Keene, NH) and ethanol precipitated twice more. The resulting fragment (approx. 1 kb) was quantitated on a gel with known standards. The fragment (25 ng) was labeled using a random primer kit (BRL, Gaithersburg, MD) and '32[P] dCTP (NEN, Boston MA).

DNA and RNA gel blots. Plum leaf DNA (6 µg) was digested with *Bam*HI or EcoRI according to manufacturer's instructions (BRL, Gaithersburg, MD) and resolved on a 0.7% agarose gel in a Probe Tech I apparatus (Oncor, Gaithersburg, MD) along with digested plasmid DNA (pGA482GG/PPV-CP-33) and known amounts of the PCR-generated PPV-CP gene fragment. The DNA was transferred, prehybridized with Hybrisol I (Oncor-50% formamide, 10% dextran sulfate, 5X SSC), and hybridized 16 h with the 32P-labeled-random primed PPV-CP fragment at 42 °C, Hybridized blots were washed three times with 0.1 X SSC at room temperature and then washed twice with 0.1X SSC at 65 °C for 30 minutes each. The blots were exposed to x-ray film with two intensifying screens at –80 °C.

Total RNA (5 µg) was resolved on agarose containing 1.4% hydroxy-methyl mercury (Bailey and Davidson 1975), transferred electrophoretically to Nytran (Schleicher and Schuell), stained with methylene blue to verify evenness of transfer and loading (Herrin and Schmidt 1988), prehybridized in Hybrisol at 42 °C and hybridized for 16 h with 32P-labeled-random primed PPV-CP fragment. The blots were washed as above, but with a final wash of 0.1 X SSC at 52 °C and exposed to film with two intensifying screens at –80 °C.

Immuno-blot assays. Young leaves from greenhouse grown plants were harvested (approx. 500 mg) and ground in 1 ml of buffer as described by Berger et al. (1989). Extracts were centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatant was collected. Total soluble protein of each sample was determined as described by Bradford (1976). Leaf protein extracts (100µg of protein) were then assayed by western blotting using polyclonal antibodies against PPV (Ravelonandro et al. 1992).

VIII. Characterization of the DNA Inserted Into C5.

DNA-blot Analyses. DNA was isolated from C5 and four other putatively transformed plants (C2, C3, C4 and C6) as well as an untransformed plum plant ('Bluebyrd'). The DNA was digested with *Bam*HI to generate a 1.2 kb fragment that would hybridize with the PPV-CP probe. The results of the DNA blot experiment are shown in **Fig. 4**. The plasmid pGA482GG/PPV-CP-33 and plants C2-C5 had the predicted 1.2 kb fragment. C5 also had a second unique sized fragment suggesting a rearrangement. The insert DNA concentrations represent approximately 1 gene copy (2 pg) to 6 gene copies (12 pg) per haploid genome. The intensity of the signal from the transformed plants suggested that there were from one to four copies of the PPV-CP gene in C5. The control plant DNA ('Bluebyrd') had no visible fragment that hybridized to the PPV-CP gene probe.

The DNA was also digested with *Eco*RI to generate one fragment that would contain part of the PPV-CP gene (approximately 7 kb) and a fragment of the plant

genome the size of which would depend upon where the T-DNA had integrated (**Fig. 4**). The DNA from each of the transformed plants exhibited at least one unique fragment. No signal was detected in the control DNA. C5 had the expected 7 kb fragment. The multiple fragments detected in C5 again indicated several insertions.

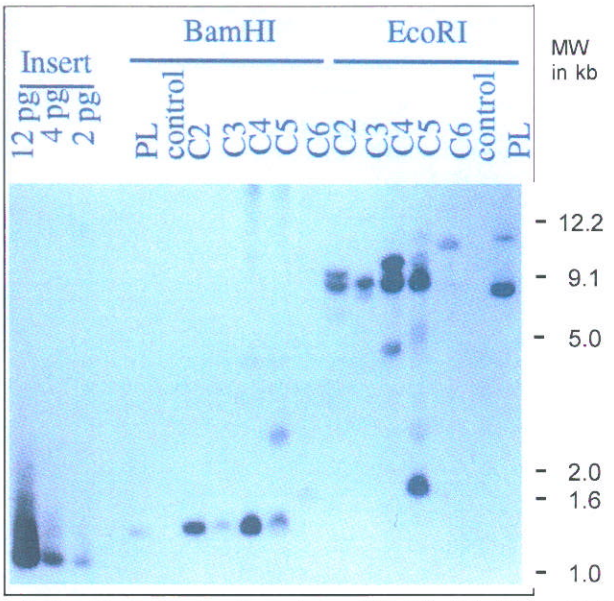


Figure 4. DNA gel blot analysis of transgenic plum clones C2, C3, C4, C5, and C6. Known amounts of the PCR-generated PPV-CP gene fragment were also resolved on the same gel. The 1.2 kb *Bam*HI fragment detected in the plasmid (PL) and in C2, C3, C4, and C5 represents the expected internal *Bam*HI fragment of the CP. The *Eco*RI digests demonstrate that C5 had unique multiple bands representing different insertion events. PL=pGA482GG/PPV-CP-33 plasmid DNA: control=untransformed 'Bluebyrd' plum.

In order to more fully investigate the structure of the PPV insert in clone C5, DNA was digested with the restriction enzymes *Eco*Rl, *Hind*III and *Bam*Hl. DNA blots were hybridized with the 1.0 kbp fragment from the PPV-CP gene, the 1.1 kbp fragment from the *npt*II gene, and the 0.8 kbp fragment from the *uid*A gene (**Fig. 1**). Assuming a complete border to border T-DNA integration of the PPV-CP cassette, an *Eco*Rl digestion probed with either *uidA* or PPV-CP sequences would reveal a 7 kbp fragment (**Fig. 1**). An *Eco*Rl digest probed with the PPV-CP sequence would produce a fragment of at least 3 kbp resulting from a cut outside of the right border of the insert. This fragment length and number would be variable depending on the location and number of inserts. The *npt*II probe also would hybridize to this > 3 kbp fragment.

When the *Eco*Rl digestion of C5 DNA was hybridized with the PPV-CP sequence five fragments were produced: a 5 and a 10 kbp fragment that corresponded to the predicted > 3 kbp fragment (these two fragments also hybridized to the *npt*II probe as predicted), and the predicted 7 kbp fragment in common with *uidA* (**Fig. 5 lane 1**). In addition to these three predicted fragments, two fragments, unique to the PPV-CP probe, were observed at 3 and 1.9 kbp (**Fig. 5, lane 1**). These last two fragments would not be expected if complete duplication of the PPV-CP cassette with intact *Eco*Rl sites had occurred. These fragments may be explained by a separate insertion or a duplication of the PPV-CP gene insert that is unlinked to the *npt*II and *uidA* gene inserts. Hybridization

with the *npt*II probe revealed a unique 20 kbp fragment that did not hybridize with either the *uidA* or the PPV-CP probe (**Fig. 5, lane 2**). This fragment may have resulted from a separate insertion or duplication of the *npt*II gene. Hybridization of the *Eco*Rl digest with the *uidA* probe showed, in addition to the expected 7 kbp fragment, a smaller than predicted 5 kbp fragment, which could have resulted from a truncation of the *uidA* gene (**Fig. 5, lane 3**). Apparent truncation of the *uidA* gene at the left border of the insert has previously been reported using this particular T-DNA vector (Scorza et al., 1995).

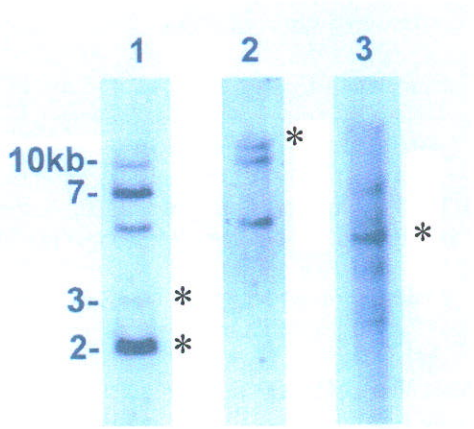


Figure 5. DNA blot analysis of transgenic clone C5 genomic DNA digested with *Eco*RI. Lane 1 was hybridized with a 1.0 kbp PCR-generated PPV-CP fragment; lane 2, hybridized with a 1.1 kbp PCR-generated nptII fragment; lane 3, hybridized with a 0.8 kbp PCR-generated *uidA* fragment. Asterisks indicate unique fragments not expected from a single insertion of the transgene construct. Fragment sizes indicated on left were derived from molecular weight standards.

When hybridized with the PPV-CP probe, blots of *Hind*III-digested DNA revealed the expected 2 kbp fragment, and *Bam*Hl-digested DNA showed the expected 1.2 kbp PPV-CP fragment (see map in **Fig. 1**). However, larger fragments also hybridized with the PPV-CP probe. One of these larger than expected fragments following digestion with *Hind*III can be seen in **Fig. 9**, PPV-CP lane 1. The large fragments are possibly the result of restriction site methylation, mutation, deletion, or rearrangement of the restriction site.

Sequencing the transgene insert in C5

In view of the extraordinarily high level of resistance of C5 to plum pox virus and the complex nature of the insert sequencing of the insert was undertaken to further elucidate its structure. Sequencing was undertaken utilizing a bacterial artificial chromosome (BAC) library developed from C5.

MATERIALS AND METHODS FOR BAC LIBRARY CONSTRUCTION AND INSERT NUCLEOTIDE SEQUENCING

High-molecular-weight DNA was extracted in solution from leaves of transgenic plum C5 by a method similar to that outlined in Georgi et al. (2002). Specifically, 25 g of frozen leaf tissue was ground in liquid Nitrogen with a mortar and pestle, suspended in 200 ml of ice cold 1 × HB (0.5 M sucrose, 10 mM Tris base, 80 mM KCl, 10 mM EDTA, 1 mM spermidine, 1 mM spermine, pH 9.4) plus 0.15% beta-mercaptoethanol and 0.5% Triton X-100. This material was squeezed through two

layers of cheesecloth and then through one layer of Miracloth (Calbiochem) into a cold 250-ml centrifuge bottle. The bottle containing the suspension was incubated on ice for 20 min. Nuclei were recovered by centrifugation at 3,300 rpm in a Sorvall GSA rotor for 20 min at 4 °C and the supernatant was discarded. The nuclei were resuspended in 1 ml of ice cold 1 × HB/BME plus Triton using a paint brush and transferred into a 50-ml Oakridge tube with an additional approximately 30 ml of the HB-Triton solution. Nuclei were recovered by centrifugation at 3,900 rpm in a Sorvall SS34 rotor for 15 min at 4 °C (this washing step was repeated until the supernatant was no longer green). The washed nuclei were suspended as before in 1 ml of 1 × HB/BME without Trition, then lysed by the addition of an equal volume of lysis buffer (50 mM Tris pH 8, 10 mM EDTA, 2% Sarkosyl). Cesium chloride was dissolved in the lysate (0.97 g of CsCl per ml of lysate). The solution was transferred to a Corex tube, centrifuged in a Sorvall SS34 rotor at 8,000 rpm for 20 min at 4 °C to remove protein, and subsequently transferred to an ultracentifuge tube and centrifuged for 2 days at 20 °C and 175,000 g. No ethidium bromide was used in the gradient. Fractions were collected from the gradient using cut-off pipette tips to avoid shearing the DNA. DNA-containing fractions were identified by diluting 2 µl of each fraction in 3 µl of water and electrophoresis on a 0.8% agarose gel. Positive fractions were dialyzed against TE (10 mM Tris, 1 mM EDTA pH 8) to remove Cesium chloride. DNA was concentrated by ethanol precipitation and resuspended in TE. This solution was stored at 4 °C.

DNA was partially digested with *Sau*3AI by incubating ca 1 µg of DNA (estimated from Ethidium bromide stained gel) with 0.04 units of enzyme for 20 min at 37 °C, then incubating for 20 min at 65 °C and adding 1/10 vol 0.5 M EDTA pH 8.0 to inactivate the enzyme. The digested DNA was electrophoresed on a conventional mini-agarose gel (0.5% SeaKem GTG agarose in 1 × TAE, 40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 97 V for 1 h at room temperature, using autoclaved running buffer. Before use, the gel apparatus was cleaned with 2% abSolve (NEN Research Products) and rinsed with sterile distilled water. Marker lanes were cut from the gel and stained with Ethidium bromide; notches were cut in the stained gel to mark the migration of uncut lambda DNA (approximately 50 kb). The stained and unstained parts of the gel were aligned and a gel slice containing fragments larger than ca. 50 kb was cut from the unstained part of the gel. These fragments were electroeluted from the gel into dialysis tubing for 0.5 h, concentrated by Centricon 100 (Millipore) spin dialysis, and ligated into pBeloBAC11. Ligation reactions contained approximately 50 ng of vector in an estimated two- or four-fold molar excess over insert DNA, and 9 Weiss units of T4 DNA ligase (NE Biolabs) in a 100-µl reaction volume, and were incubated overnight at 8 °C. Ligation products were introduced into *Escherichia coli* strain DH10B (Electromax, Invitrogen) by electroporation using a BioRad Genepulser set to 2.5 kV, 200 Ω, 25 µF, and 0.2 cm gap cuvettes. The cells were added to 1 ml of SOC and incubated, shaking, at 37 °C for 1 h; then a portion of the transformation was plated on selective media (LB plus chloramphenicol, IPTG and X-Gal). Selected clones were grown up and their DNA extracted (see method below) and electrophoresed undigested, to verify the presence of large inserts prior to transforming and plating large numbers of clones for the library. Plates were grown overnight at 37 °C, then held for an additional day in the dark at room temperature to permit the color to develop sufficiently for detection by the robot. The resulting clones were robotically arrayed by a Genetix Q-Bot into 384-well plates and stored at –80 °C.

Hybridization

Clones from the BAC library plates were robotically arrayed in duplicate on five 22 × 22 cm Hybond N+ (Amersham) filters for hybridization. One library filter set was hybridized with PCR products of NPTI and GUS genes. Probes were labelled

with α^{32}P dCTP by the method of random priming. After 2 h of prehybridization at 65 °C in 7% SDS, 0.25 M sodium phosphate buffer (pH 7.4), hybridizations were carried out overnight in a fresh charge of the same solution. Filters were then washed twice at the hybridization temperature, each time for 15 min, in 2 × SSC, 0.1% SDS; and two times (15 min each) in 0.3 × SSC, 0.1% SDS. Hybridization signals were detected autoradiographically using Kodak XAR film.
BAC DNA prepared from positive clones was digested with *Eco*RI, *Bam*HI or *Hin*dIII, electrophoresed on 0.8% SeaKem LE agarose and stained with ethidium bromide. Southern transfer of the DNA to Hybond N+ membranes was performed using the manufacturer's Alkaline Transfer Protocol. Southern blots were hybridized with the appropriate probe as described.

BAC DNA extraction
BAC DNA was extracted by a modified alkaline-lysis procedure: clones were grown overnight in 5 ml of LB broth and harvested by centrifugation in a Beckman GPR centrifuge, GH-3.7 swingout rotor for 10 min at 4 °C. Pelleted cells were resuspended in 0.3 ml of GTE (50 ml of glucose, 25 mM Tris pH 8, 10 mM of EDTA pH 8) containing 0.1 mg/ml of RNase A, transferred into microfuge tubes, and lysed by adding 0.3 ml of 0.2 N NaOH/1% SDS and incubating at room temperature for 2.5 min. The lysates were neutralized by adding 0.3 ml of 3.0 M potassium acetate, pH 4.8. Following a 5-min incubation, cellular debris was removed by centrifugation in a microfuge for 5 min at room temperature. BAC DNA was precipitated by adding 0.7 vol of isopropanol. Larger-scale BAC DNA extractions (50–500 ml culture volume) were additionally purified on cesium chloride density gradients using a Beckman TL100 ultracentrifuge.

Subcloning and sequencing
For sequencing, BAC DNA was digested with *Sau*3AI, *Eco*RI, *Bam*HI or *Hin*dIII, and the resulting fragments were ligated into pUC119 or pBluescript and transformed into *Escherichia coli* strain DH5α by calcium/heat shock. Plasmid DNA for sequencing was prepared using a protocol very similar to the one described above for BAC DNA, followed by precipitation from 0.4 M NaCl, 6.5% PEG. Subclones were sequenced using ABI's Dyedeoxy terminator cycle sequencing kit and an ABI377 DNA sequencer. Additional sequencing was performed by the USDA-ARS ERRC Nucleic Acids Research Faciliity, Philadelphia, PA. Sequences were assembled using Sequencher 4.2 software (GeneCodes Corp.).

Summary of C5 insert sequencing.
Due to sequence repeats, DNA methylation, and the presence of an origin of replication in the insert, sequencing was difficult and for some parts of the insert, inconclusive. With the methods outlined above we have sequenced approximately 80% of the insert. Combining the information obtained by restriction mapping, sequencing, and PCR, a 5 part structure of the insert is proposed (**Fig. 6**) with all parts in close proximity in the C5 plum genome behaving in terms of inheritance as a single locus (see section XI: Inheritance of the transgene and plum pox virus resistance in the progeny.) This 5-part structure contains transgene duplications, rearrangements, and importantly, an inverted repeat of the PPV-CP gene which may be critical for providing PPV resistance.

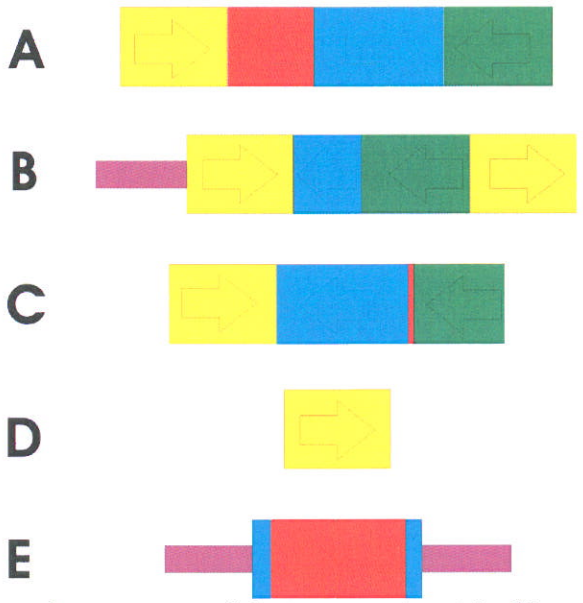


Figure 6. Proposed structural components of the transgene insert in C5 as determined by DNA blot analyses and sequencing. Yellow=NPTII, red=PPV-CP, blue=GUS, green=sequence from pBR322, purple=plum DNA sequences. Genetic analyses have suggested that these insert pieces, A through E, are linked. The precise distance, one from another is not known.

C5 contains the following transgene fragments as illustrated in **Fig. 6**:

A. A complete insert consisting of the NPT, PPV-CP and GUS genes and their respective promoters plus sequence originating from pBR322 (see **Fig. 1**).
B. A fragment consisting of the NPTII gene with the nopaline synthase (nos) promoter and part of the GUS gene, the pBR322 segment and another copy of the NPTII gene with nos promoter, bordered at the 5' position by plum DNA.
C. The NPTII gene, the GUS gene with a small segment of the 35S promoter from the PPV-CP gene, and part of the pBR322 segment.
D. A single copy of the NPTII gene (it is not known if the nos promoter is present).
E. A 3'-3' tail-to-tail copy of the PPV-CP with the 35S promoter for each copy and a portion of GUS sequence flanking each PPV-CP copy. The insert flanked by plum DNA.

Interruption and inactivation of the β-lactamase (ampicillin resistance) gene

Sequencing revealed that the β-lactamase gene in the transgene insert in C5 is interrupted by a fragment containing bacterial *cos* site (**Fig. 1**) presumably inactivating this antibiotic resistance gene. In order to address the inactivation hypothesis, expression of the β-lactamase gene in C5 was investigated. RNA was extracted from frozen (-80 °C) leaf samples of field grown C5 trees from October 1997, July 2000, April 2002, July 2002, May 2003, July 2003, October 2003, November 2004 and May 2005 and from greenhouse-grown C5 from December 2005. Reverse transcriptase (RT)-PCR using a forward primer beginning at position 308 in β-lactamase gene and a reverse primer beginning at position 840 spanning the *cos* insertion site was used. Results of this study clearly demonstrated that no β-lactamase mRNA was produced (**Fig. 7A**). Further, we addressed the possibility of deletion of the *cos* site that might lead to the activation of the

β-lactamase gene through in vitro studies with the plasmid used to produce C5. *Escherichia coli* strain DH5α transformed with plasmid pGA482GG-PPVcp and *E. coli* DH5α without a plasmid were cultured overnight in liquid LB medium containing 50 mg/L gentamycin or without antibiotics, respectively. Cell density was determined by titration on the same media with the addition of agar. $1x10^9$ cells of *E. coli* DH5α transformed with pGA482GG-PPVcp containing a disrupted β-lactamase gene and DH5α without a plasmid were plated on LB medium containing 100 mg/L ampicillin. In addition, DH5α containing an expression vector pUC18cpexpphas with a functional β-lactamase gene and the PPVcp gene were also plated on LB medium containing 100 mg/L ampicillin.

From the plating of $1x10^9$ cells of DH5α/pGA482GG-PPVcp five colonies grew on LB medium containing 100 mg/L ampicillin, whereas no colonies were obtained from the same quantity of DH5α without a plasmid. A replication of this trial produced identical results. PCR analyses of plasmid prepared from liquid cultures of the five amp resistant DH5α/pGA482GG-PPVcp colonies indicated that the *cos* site inserted in the β-lactamase gene had excised completely, partially and not at all in an apparently mixed bacterial cell population. (**Figure 7B**)

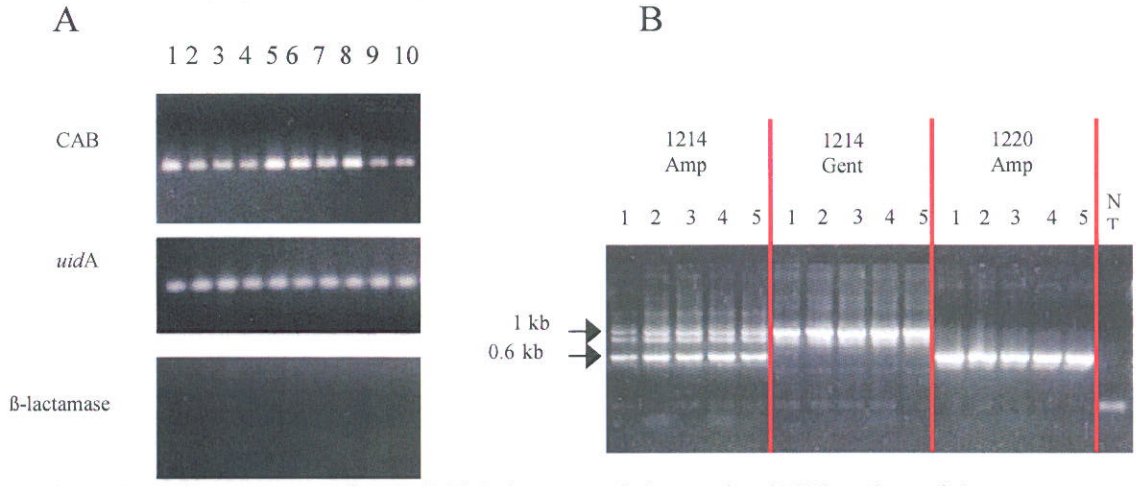


Figure 7. **A.** Reverse transcriptase (RT) Polymerase chain reaction (PCR) to detect β-lactamase gene mRNA in C5. The RNA was extracted from leaves of field grown C5 (lanes 1-9) and C5 growing in the greenhouse (lane 10). Sample dates: lane 1- Oct 1997. lane 2- July 2000. lane 3- April 2002. lane 4- July 2002. lane 5- May 2003. lane 6- July 2003. lane 7- Oct 2003. lane 8- Nov 2004. lane 9- May 2005. lane 10- Dec 2005. The chlorophyll a/b (CAB) gene mRNA and the gus (*uid*A) transgene mRNA were both detected while the β-lactamase transgene mRNA product was not detected in these samples. The β-lactamase gene was detected in *E. coli* control samples run separately (data not shown). This work determined that C5 does not express the β-lactamase gene. **B.** PCR detection of *cos* site insertion in the β-lactamase gene contained in *E. coli* DH5α/pGA482GG-PPVcp. The upper approximately 1 kb band is indicative of the β-lactamase gene containing the *cos* insert. The approximately 0.6 kb band indicates the absence of the *cos* insert. The intermediate band present in lanes 1 to 5 of 1214 Amp could result from a partial deletion of the *cos* insert. Bacterial Strain #1214 Amp: DH5α/pGA482GG-PPVcp grown on ampicillin, Bacterial Strain #1214 Gent: DH5α/pGA482GG-PPVcp grown on gentamycin, Bacterial Strain #1220 Amp: DH5α/pUC18cpexpphas, NT: No Template Control. The forward primer begins at position 308 in β-lactamase gene, the reverse primer begins at position 840 in β-lactamase gene. The PCR product spans the *cos* insert site. This work indicates that of 1 million *E. coli* DH5α colonies produced containing the insert shown in **Fig. 1**, five appeared to lose the interrupting *cos* site.

While C5 contains several copies of the DNA region originating from plasmid pBR322 that contain the β-lactamase gene, the gene is interrupted by a fragment containing the *cos* site and we have shown that in C5 leaf samples spanning 8 years of tree growth in the field the gene is non-functional. The potential for mutation in which the *cos* site could be eliminated to produce a functional β-lactamase gene was tested in *E. coli* and found to occur at a rate of 5 events in 1,000,000,000 *E. coli* cells.

Stability of the structure of the transgene insert in C5. The structure of this insert has been repeatedly evaluated throughout the years from 1990 to 2005 through EcoR1 digests and DNA blotting using PPV-CP, NPTII and/or GUS probes. These tests have indicated that the insert is stable in both vegetatively propagated plants of C5 and progeny of C5 carrying the insert.

IX. Basis of Resistance in C5 – Investigations of Post-transcriptional Gene Silencing (PTGS)

Nuclear Run-On Analysis. To determine if the reduced levels of PPV-CP mRNA in clone C5 were due to post-transcriptional regulation of the transgene, nuclear run-off transcription analysis was performed. Clone C5 (low levels of PPV-CP mRNA), clone C4 (high levels of PPV-CP mRNA), and a non-transformed control were analyzed. Transcription of PPV-CP (**Fig. 8, all lanes 2**) was compared with the transcription of actin (**Fig. 8, all lanes 1**). The non-transformed control produced the actin transcript but not the PPV-CP transcript, as was expected. The levels of both actin and PPV-CP transcripts were at similarly high levels in clones C4 and C5, indicating that the low level of PPV-CP mRNA that accumulated in C5 was apparently due to post-transcriptional down-regulation.

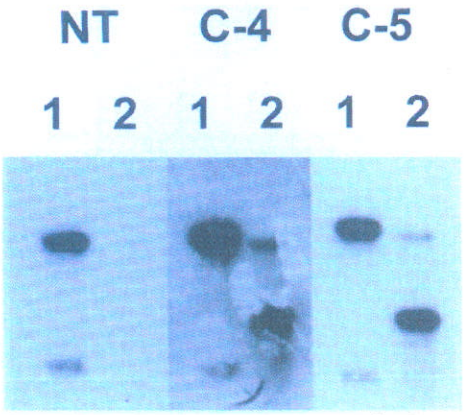


Figure 8. Nuclear run-on assay of non-inoculated PPV-CP transgenic clones C4 and C5. NT lane is non-transformed control plum. Lane 1= 2 μg of actin plasmid digested by *Xho*I. Lane 2= 2 μg of PPV-CP plasmid double digested with *Bam*HI and *Asp*718 (Scorza et al., 2001).

Transgene methylation in clone C5. Transgene methylation has been shown to be associated with PTGS (Ingelbrecht et al., 1994; English et al., 1996; Davies et al., 1997; Guo et al., 1999; Jones et al., 1999; Kohli et al., 1999; Sonada et al., 1999). In order to evaluate methylation of the transgene insert in clone C5, DNA was digested with

methylation sensitive (*Alu*I, *Bam*HI, *Hind*III, *Sau*3A) and methylation insensitive (*Mbo*I, *Hin*fI) enzymes. The digests were split into 4 subsamples and hybridized with probes made to the PPV-CP, *npt*II, *uid*A genes and to the CaMV 35S promoter region. The same digests were also subjected to PCR, following the strategy of Ingelbrecht et al (1994), using the primers that were used to construct the probe sequences. **Figure 9** presents the results from one such experiment using DNA from the silenced C5 and the non-silenced C3 lines. The DNA was initially digested with *Hind*III and then with either *Mbo*I or *Sau*3A. Evidence of methylation is seen in the PPV-CP sequence in C5. This is indicated by the multiple, larger than expected-sized fragments in the *Sau3*A digest as compared to the *Mbo*I digest when probed with PPV-CP (**Fig. 9 PPV-CP, lane 3**). No differences were seen in C3 between the *Mbo*I and *Sau*3A digests (**Fig. 9 all lanes 5 and 6**) nor in the C5 when hybridized with *uidA*, *npt*II or CaMV 35S sequences (**Fig. 9 lanes 2 and 3 of the respective probes**). These results indicated specific methylation of the PPV-CP insert, lack of methylation of the *uidA*, *npt*II and CaMV 35S sequences, and complete digestion of the sampled DNA. The PCR reactions for the same digests (**Fig. 9**) supported the DNA blotting indicating that the C5 PPV-CP but not the *npt*II or the *uidA* transgenes were methylated since PCR of the *Sau*3a digest using PPV-CP-specific primers generated product only in clone C5 (**Fig. 9, PPV-CP lane 3**). Additionally, although DNA blotting did not indicate methylation of the CaMV 35S promoter region in C5, PCR results, which are more sensitive than DNA blotting, indicated a low level of methylation in C5 (**Fig. 9, CaMV 35S PCR lane 3**).

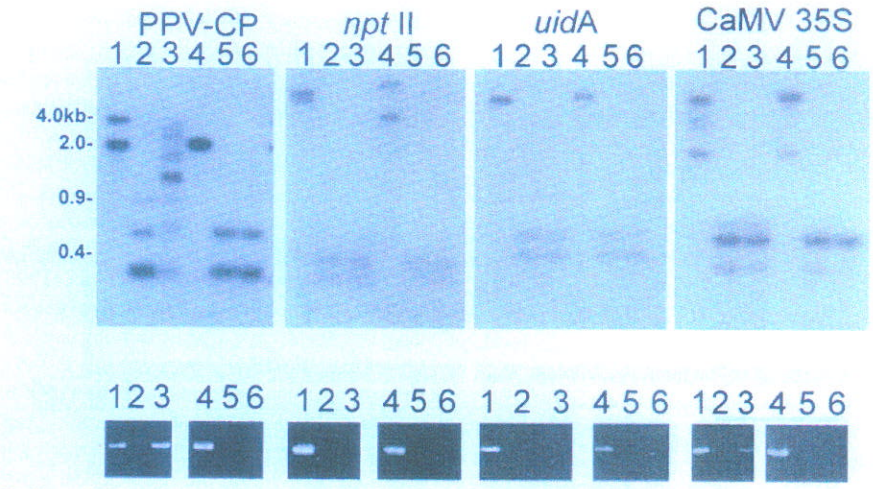


Figure 9. DNA blot and PCR analysis of genomic DNA from transgenic clones C5 (silenced) and C3 (non-silenced) demonstrating methylation status of the PPV-CP, *npt*II, and *uid*A genes, and CaMV 35S promoter. Lanes 1 – 3 are C5. Lanes 4 – 6 are C3. Lanes 1 and 4 were digested with *Hind*III, lanes 2 and 5 with *Hind*III and *Mbo*I. Lanes 3 and 6 were digested with *Hind*III and *Sau*3a. Hybridization probes and primer pairs from left to right: PPV-CP, *npt*II, *uid*A, CaMV 35S. Fragment sizes indicated on left were derived from molecular weight standards. The DNA was initially digested with *Hind*III and then with either *Mbo*I or *Sau*3A. Evidence of methylation is seen in the PPV-CP sequence in C5. This is indicated by the multiple, larger than expected-sized fragments in the *Sau3*A digest as compared to the *Mbo*I digest when probed with PPV-CP (PPV-CP, lane 3). No differences were seen in C3 between the *Mbo*I and *Sau*3A digests (all lanes 5 and 6) nor in the C5 when hybridized with *uidA*, *npt*II or CaMV 35S sequences (lanes 2 and 3 of the respective probes). These results indicated specific methylation of the PPV-CP insert, lack of methylation of the *uidA*, *npt*II and CaMV 35S sequences, and complete digestion of the sampled DNA. The PCR reactions for the same digests supported the DNA blotting indicating that the C5 PPV-CP

but not the *npt*II or the *uidA* transgenes were methylated since PCR of the *Sau*3a digest using PPV-CP-specific primers generated product only in clone C5 (PPV-CP lane 3). Additionally, although DNA blotting did not indicate methylation of the CaMV 35S promoter region in C5, PCR results, which are more sensitive than DNA blotting, indicated a low level of methylation in C5 (CaMV 35S PCR lane 3) (Scorza et al., 2001).

Methylation of the PPV-CP insert in C5 was further investigated by digesting C5 and C3 DNA with *Bam*H1 and then with the C-methylation sensitive enzymes *Alu*l, *Eco*Rl, and *Sau*3a, and methylation insensitive *Hin*fl. Comparison of the C3 and C5 digests reveals the presence of multiple, larger fragments in C5 indicating C-residue methylation (**Fig. 10, lanes 1, 3, 4**). Although, unlike methylation sensitive *Alu*l, *Sau*3a, and *Eco*Rl digestions, the unexpected fragment from the *Hin*fl digestion (**Fig. 10, lane 2**) cannot be explained by methylation. This unexpected fragment is likely the result of the aberrant multicopy nature of the C5 insert [**Fig. 6(A)**].

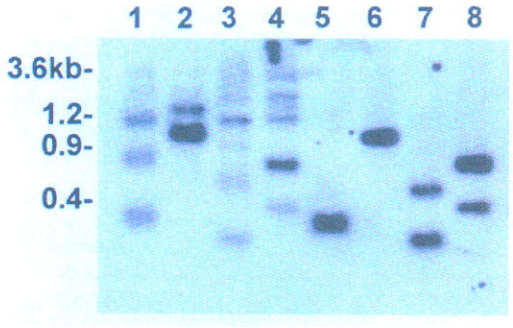


Figure 10. DNA blot analysis of genomic DNA from transgenic clones C5 and C3 demonstrating methylation of the PPV-CP gene insert: Lane 1, double-digestion of C5 with *Bam*HI and *Alu*I; lane 2, double-digestion of C5 with *Bam*HI and *Hin*fI; lane 3, double-digestion of C5 with *Bam*HI and *Sau*3a; lane 4, double-digestion of C5 with *Bam*HI and *Eco*RI; lanes 5-8, C3 digested with the same restriction enzymes as in lanes 1-4, respectively. Fragment sizes indicated on left were derived from molecular weight standards. *Alu*I, *Bam*HI, *Eco*RI, and *Sau*3a are methylation sensitive; *Hin*fI is methylation insensitive. Blots were hybridized with the 1.0 kbp PPV-CP-specific probe (Scorza et al., 2001).

siRNA Production. Perhaps the most diagnostic characteristic of gene silencing is the production of short interfering RNAs (siRNAs) (Hamilton and Baulcombe, 1999) that derive from dsRNA specifically degraded by DICER, an Rnase III-like enzyme (Bernstein et al., 2001). These siRNAs are then loaded into a multi-subunit endonuclease, the RNA-induced silencing complex (RISC) (Hammond et al., 2000), which scans the pool of messenger RNA. SiRNAs ensure that the entire target molecule (in the case of C5, viral RNA) that share sequence similarity with the former dsRNA is specifically degraded (Elbashir et al., 2001). Thus, siRNA are diagnostic for PTGS. To study the correlation of PTGS and siRNA production in C5, two parallel experiments were designed under greenhouse conditions. The first trial involved plants that were healthy, and the second trial plants that had been inoculated with *plum pox virus*. To assess whether the production of siRNAs was specifically associated with the resistant PTGS phenotype C5, we used for this study the transgenic PPV-susceptible clone C3-2 and C5, both harboring the coat protein gene of *plum pox virus* (PPV-CP). We chose as

controls, plum clone PT-23, a tree transformed only with the two marker genes, NPTII and GUS and also the untransformed susceptible commercial plum cultivar 'Bluebyrd'.

Total RNA from C5 was analyzed for the presence of siRNA with a sequence specific to the PPV-CP gene (Scorza et al, 1994). Small RNAs of approximately 22 nt and 25-26 nt were detected in nucleic acid extracts, both from healthy and PPV inoculated C5 leaves (**Fig. 11 and 12 A, lane 5h and 5i**). This indicates that C5 constitutively produces both siRNA species and that PPV inoculation is not required to induce silencing in this particular clone.

In contrast, no siRNAs were detected in healthy PPV sensitive transgenic plum C3-2, PT-23 and untransformed controls (**Fig. 11, lane Bh, Ph and 3h**). While both C5 and C3-2 contain multicopy inserts (Scorza et al., 1994), only C5 is silenced and produces siRNAs, indicating that not only the copy number but other factors, perhaps the structure of the insert copies such as a self-complementary structure, is important in producing the silenced phenotype. It has been shown that a self-complementary 'hairpin' RNA (hpRNA) construct efficiently silences genes (Wesley et al., 2001). Constitutive production of siRNA adds additional evidence to the transgene structure presented in **Figure 6** indicating that C5 contains apparently duplicated copies or partial copies of the PPV-CP with perhaps a self-complementary "hairpin" segment.

After stripping the PPV-CP probes, the membrane was hybridized with a probe specific to detect the PPV-P1 gene (**Fig. 12 B**). The same pattern of virus degradation products and siRNA (corresponding to the 22 nt class size) were observed in extracts of susceptible clones (compare **Fig. 12 A and 12 B**, lane Pi and Ci). By contrast, C5 leaf extracts (**Fig. 12 B, lane 5i**) were below detection limit, and no virus sequences nor siRNAs were detected. This figure was reproduced with a PPV-HCpro specific probes (data not presented). This confirms C5 was virus-free and that the viral PPV-CP sequence of the transgene is the source of siRNA inducing the resistant phenotype of clone C5. Interestingly, our work shows that upon inoculation with PPV, untransformed plums produce the small class of siRNA indicating that the production of siRNA, specifically the short 22 nucleotide class, is a natural consequence of PPV infection.

The detection of siRNA in post-transcriptionally silenced PPV resistant C5 plum completes the view of the process of resistance demonstrating all components of the PTGS pathway as currently described in herbaceous species. C5 constitutively express both short and long siRNA, involving both local and systemic silencing, respectively. It appears that the long siRNA may be also specifically associated with methylation (Hamilton et al, 2002). The restriction of long-distance movement of PPV in C5 when grafted onto PPV-infected rootstock-inoculated and has been confirmed by Ravelonandro in both greenhouse tests in France and in field tests in Poland (Hily et al., 2004), Romania and Spain (unpublished).

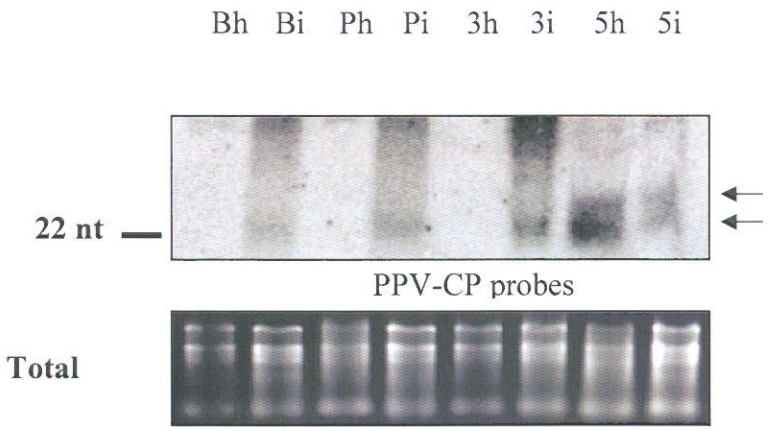


Figure 11. The effect of *Plum pox* virus inoculation on siRNAs accumulation in plum leaves. RNA gel blot analysis for detection of PTGS-typical small interfering (si) RNAs. Nucleic acid preparation from healthy (Bh, Ph, 3h and 5h) and inoculated (Bi, Pi, 3i and 5i) untransformed and transgenic plum leaves were separated on a 20% denaturing polyacrylamide gel and hybridized with radioactive DNA probes corresponding to both strand of the full length of the PPV-CP sequence (Scorza et *al.*, 1994). Position of the 22 nt DNA marker is at the left. Relative quantification was performed on a 1% non-denaturing gel using ethidium bromide staining. (B='Bluebyrd' non-transgenic control, P=PT23 transformed with *nptII* and GUS markers only, 3=PPV-CP transgenic clone C3-2, 5=PPV-CP transgenic clone C5; h=healthy, i=inoculated).

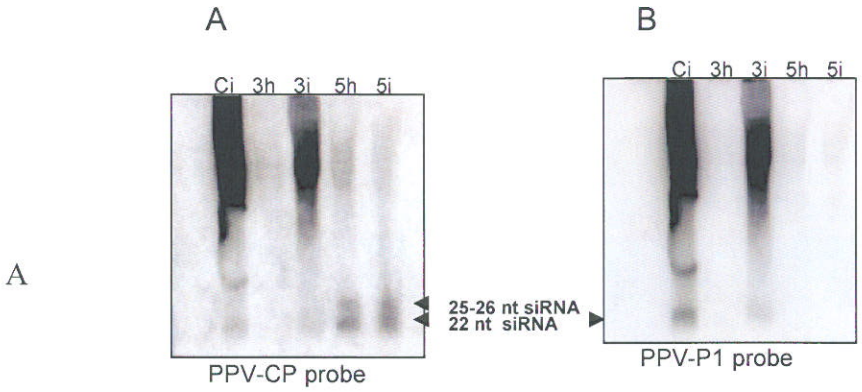


Figure 12: Accumulation of siRNAs corresponding to different region of the virus in sensitive clones. (A) Northern blot analysis to search for the PPV-CP gene specific siRNAs. Nucleic acid preparation from healthy (h) and PPV-infected (i) plum leaves were separated in a 20% denaturing polyacrylamide gel. (B) The same membrane as that in (A) after stripping and probed with PPV-P1 gene. Between hybridization, the membrane was stringently stripped (50% formamide, 2X SSPE, 1X SDS at 65°C for 90 min), control exposures confirmed the removal of the hybridization probe. Positions of the 22 and 25-26 nt DNA markers are indicated with arrows. (C=non-transformed control, 3=transgenic clone C3-2, 5=transgenic clone C5; h=healthy, i=inoculated).

MATERIALS AND METHODS FOR INVESTIGATIONS OF POST-TRANSCRIPTIONAL GENE SILENCING (PTGS)

Extraction of nucleic acids. Leaves for nucleic acid extraction were generally collected from mature, field-grown trees and were immediately frozen in liquid nitrogen and stored at –80°C. DNA, and RNA were extracted from leaf samples, as previously described (Kobayashi et al., 1998; Verwoerd et al., 1989).

DNA methylation analysis. Methylation status was evaluated, as previously described (Ingelbrecht et al., 1994; Scorza et al., 2001). Briefly, restriction

digestion of 5 μg of genomic DNA was carried out overnight at 37°C using 1 unit/μg for each enzyme (methylation sensitive Sau3A I and methylation insensitive Mbo I) (Gibco BRL, Life Technologies) in the presence of 5mM spermidine in a volume of 200 μl.

Quantitative PCR was performed using an ABI Prism 7900HT sequence detection system with 2X SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). The PCR reactions were performed in triplicate for each sample. Real time amplification plots were used to determine the threshold cycle number (Ct), which is the cycle at which a significant increase in amplification (as measured by binding SYBR® Green to specific product) is first detected (TaqMan PCR protocol, Applied Biosystems). Since cytosine methylation will inhibit Sau3A I digestion, the higher the level of methylation, the higher the amplification value. Values were quantitatively standardized to take into account the level of digestion. A dissociation curve analysis was performed after each run to screen for non-specific products. New primers were specifically designed by Primer express 2.0 software for methylation analysis by TaqMan quantitative PCR (See Hily et al., 2004).

RT-PCR. Following DNase treatment (DNase I- RNase-free, 1 unit/μg, Roche, Mannheim, Germany), RT-PCRs were conducted in a one step reaction using the GenAmp® EZrTth RNA PCR Kit (Applied Biosystems) from 100 ng of total RNA, in 25 μl of reaction according to the manufacturer's instructions. Samples were heated at 60°C for 30 min (reverse transcription phase), and after one min at 94°C, samples went through 40 cycles 94 (15 sec) – 60°C (30 sec). Following cycling, 7 min at 60°C completed the extension. Specific primers were used to detect M and D strains of PPV (previously described by Olmos et al., 1997). Also, specific primers to distinguish viral from transgene PPV-CP mRNA were used as described in Ravelonandro et al. (1992). RNA quality was verified by amplification of 18S ribosomal RNA.

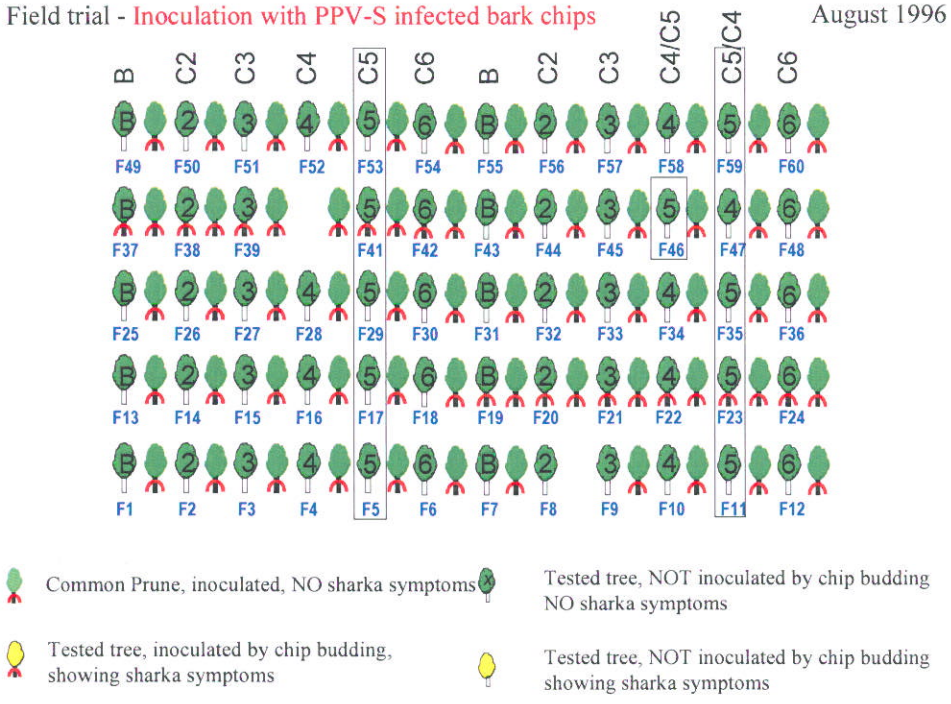
siRNA Detection. Total RNA from leaves were extracted using the purescript kit (Gentra systems, Inc., Minneapolis, MN). The lysis buffer was amended with 2% w/v PVP-40 (Invitrogen, Carlsbad, CA), per the manufacturer's recommendation, for woody plant RNA isolation. Samples were then redissolved in 50% formamide. Relative quantification of low molecular weight RNA was performed on a 1% non-denaturing gel using ethidium bromide staining. Samples (≅20μg of total RNA) were heat treated and loaded onto a 0.5X Tris-Borate-EDTA (TBE) polyacrylamide gels (20%). DNA oligonucleotides ranging from 22 to 27 nt were also loaded onto the gel as size markers. After electrophoresis, the nucleic acids were electroblotted in 0.5X TBE buffer to a Hybond Nx membrane (Amersham Biosciences, Buckinghamshire, UK) and fixed by UV irradiation. Hybridization was performed using in vitro synthesized ^{32}P-labelled DNA transcripts generated as previously described (Scorza et al., 1994).

X. Inheritance and Stability of the Introduced Functional Trait – *Plum Pox Virus* Resistance in the C5 Plant.

Evaluation of the stability of PTGS and plum pox virus resistance. In order to evaluate the long-term stability of the PPV-CP transgene in clone C5, field trials were established beginning in 1996-1997, under the appropriate European permits, in Poland (Polish Ministry of Environment permit number 8/2002), Spain (Permission B/ES/96/16 by the Spanish Ministerio de Medio Ambiente), and Romania (local institutional authorization). The specific procedures and methodologies used in the Poland and Spain tests will be described in detail here. The Romania test will be briefly presented since it serves to

corroborate the results from the Poland and Spain tests. Reports of these tests have been published in Hily et al., 2004 (Poland); Malinowski et al., 1998 (Poland); Ravelonandro et al., 2002b (Romania); Malinowski et al 2006 (Poland, Spain) .

Poland field test. In Poland, five transgenic plum clones (C2, C3, C4, C5, C6), previously described (Scorza et al., 1994; Ravelonandro et al., 1997, 1998a) and untransformed control plum B70146, all grafted onto GF8-1 rootstocks, were planted in April 1996 in a newly established field trial at the experimental field of the Research Institute of Pomology and Floriculture near Skierniewice, Poland (Malinowski et al., 1998). In August 1996, two of ten trees of each clone were inoculated by chip bud inoculation (CBI) as described by Malinowski et al. (1998) with a PPV-D isolate of PPV that was described from Poland and is referred to as PPV-S (Skierniewski) (Wypikewski et al. 1994). The other eight trees of each clone were left for inoculation through aphid vectored inoculation (AVI). Trees of 'Sweet Common Prune' (syn. 'Hauszwetsche') were planted in rows alternating with rows of test trees. These 'Sweet Common Prune' trees were CBI with PPV-D to provide ample sources of inoculum for aphid acquisition of PPV [**Fig. 13 (A)**]. Also, naturally PPV-infected plum trees grown in close vicinity of the field test (within 50 m) provided additional sources of inoculum.



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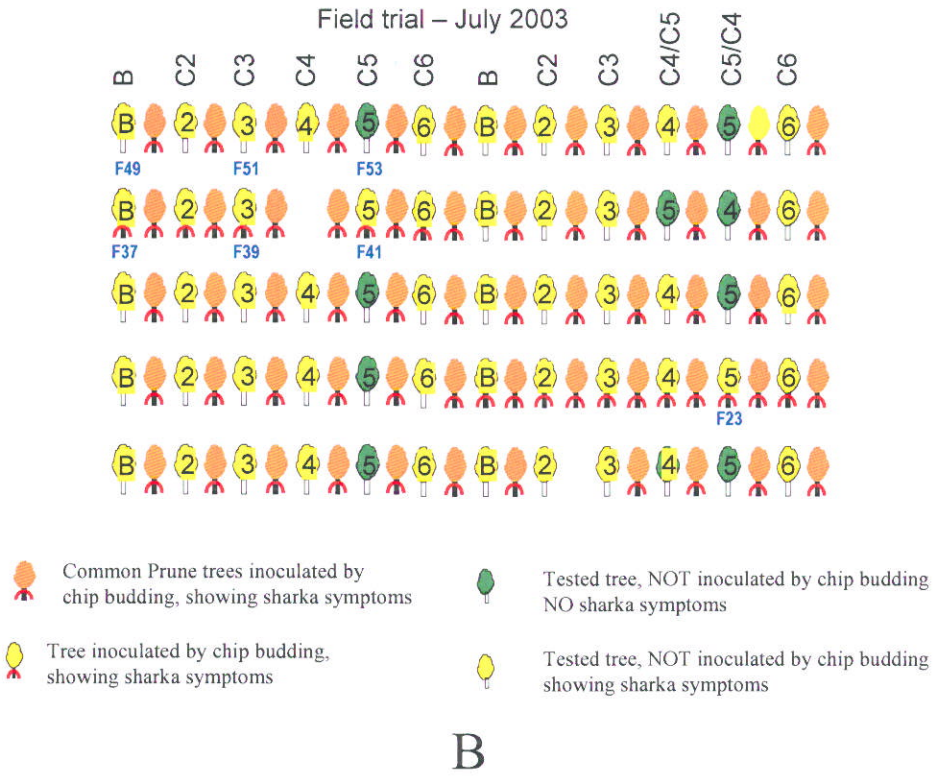

Figure 13. (A) Field plot of PPV-CP transgenic plum field trial 1996 and (B) in 2003. Trees were inoculated in August 1996. five transgenic plum clones (C2, C3, C4, C5, C6), previously described (Scorza et al., 1994; Ravelonandro et al., 1997, 1998a) and untransformed control plum B70146, all grafted onto GF8-1 rootstocks, were planted in April 1996 in a newly established field trial at the experimental field of the Research Institute of Pomology and Floriculture near Skierniewice, Poland (Malinowski et al., 1998). In August 1996, two of ten trees of each clone were inoculated by chip bud inoculation (CBI) as described by Malinowski et al. (1998) with a PPV-D isolate of PPV that was described from Poland and is referred to as PPV-S (Skierniewski) (Wypikewski et al. 1994). The other eight trees of each clone were left for inoculation through aphid vectored inoculation (AVI). Trees of 'Sweet Common Prune' (syn. 'Hauszwetsche') were planted in rows alternating with rows of test trees. These 'Sweet Common Prune' trees were CBI with PPV-D to provide ample sources of inoculum for aphid acquisition of PPV. Also, naturally PPV-infected plum trees grown in close vicinity of the field test (within 50 m) provided additional sources of inoculum. Trees numbered in the July 2003 field map were those studied in detail by Hily et al. (2004). The field plots indicate that all trees of became infected by 2003 except C5 trees left to natural aphid inoculation. These results are also consistent in 2004.

Evaluation of PPV infection. PPV infection of test trees was evaluated by visual observation of symptoms, ELISA, RT-PCR or IC-RT-PCR. Briefly, symptoms were evaluated each month throughout the growing season. Standard double-antibody sandwich ELISA for PPV was performed two to three times per growing season. RT-PCR amplification of the fragment of the PPV nuclear inclusion body (NIb) cistron using the primers PPV-A, PPV-B was performed when necessary to confirm ELISA results. IC-RT-PCR with the same primer pair was also used to confirm the absence of infection in some trees (Hily et al., 2004).

After 7 years, all eight trees of clone C5 exposed to aphid vectored infection (AVI) remained healthy in the field based on symptomology, ELISA and IC-RT-PCR analyses [**Fig.13 (B)**]. These results confirmed greenhouse tests (Ravelonandro et al., 1997, 1998a). In contrast, all trees of other clones became infected under the same conditions. Susceptible transgenic clones and non-transformed trees in the test block, both CBI and those trees left to AVI, began exhibiting symptoms the first summer after inoculation or exposure to aphids in the field, with an increasing number of trees becoming infected yearly. By the end of the fourth year in the field, 95% of these sensitive trees were infected as determined by symptom expression (**Fig. 14a and 14b**) and ELISA (**Fig. 14d**) and by year seven all sensitive trees were infected. Although highly resistant, C5 trees were not immune to PPV. The two C5 trees that were CBI displayed mild symptoms (**Fig. 14c**) on a few leaves on single branches starting from the second year after inoculation. Symptoms appeared on CBI C5 one year later than for the other CBI trees. By year four, CBI C5 trees produced no symptomatic leaves, but during the growing season a few samples were ELISA positive. ELISA readings were nevertheless consistently lower for CBI C5 than for susceptible clones whether the susceptible clones were CBI or AVI (**Fig. 14d**). The June 2000 CBI C5 tree samples tested were ELISA and RT-PCR negative for PPV, but PPV was detected by IC-RT-PCR (compare **Figs. 14d, 14e, 14f and 15b, 15c**) generally on symptomatic leaves or in a few cases on asymptomatic leaves from branches that produced symptomatic leaves in the previous year.

Analyses of symptom development, ELISA, and molecular determinations of infection separated clones in this field trial into three groups: I) A highly resistant class consisting of C5 with no apparent aphid vectored infection (AVI). CBI C5 trees displayed very mild and delayed symptoms (**Fig. 14c**), were generally ELISA and RT-PCR negative (**Fig. 14d, 14f, 15b and 15c**), IC-RT-PCR positive (2 of 6 samples for both C5 CBI trees, **Fig. 14e**). (II) Susceptible C2, C3, C4, and C6 with all showing symptoms after seven years and all trees ELISA and IC-RT-PCR positive. (III) Highly susceptible B70146 displaying severe chlorotic symptoms visible from a distance after only one to two years following inoculation (**Fig. 14a**), ELISA (**Fig. 14d**), and IC-RT-PCR positive (data not shown). This grouping of clones followed the classification expected from greenhouse trials (Ravelonandro et al., 1997, 1998a).

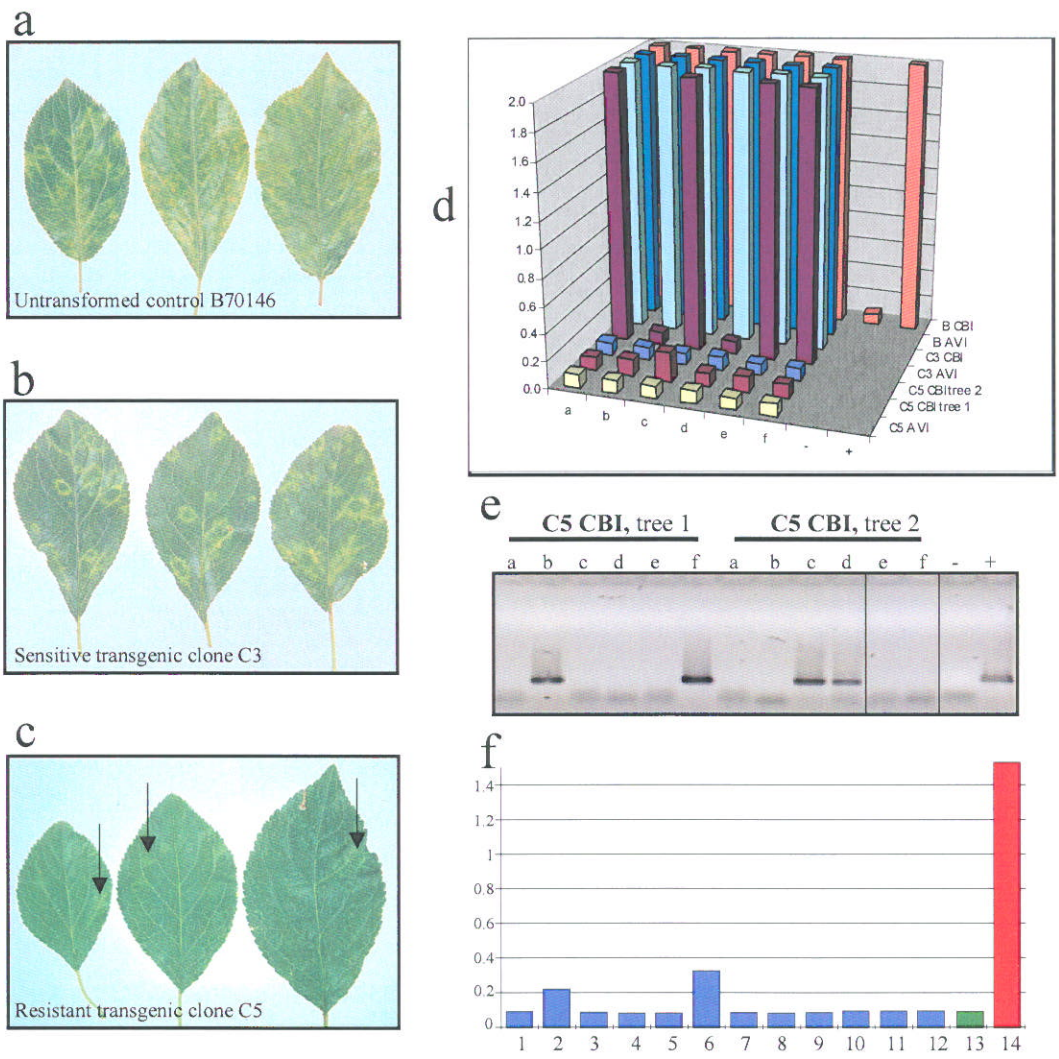

Figure 14. Detection of *Plum pox virus*. (a) Symptoms of PPV infection on CBI untransformed clone leaves, (b) CBI susceptible transgenic C3 clone and (c) very mild symptoms on CBI C5 (indicated by arrows). (d) ELISA analysis of six separate leaf samples for each tree (Sampled on 6/6/2000). Simultaneous (e) Immuno-capture Reverse Transcriptase Polymerase Chain Reaction (IC-RT-PCR) and (f) Enzyme-Linked-Immunosorbant-Assay (ELISA) analysis of both CBI C5 trees (Sampled on 6/13/2000). The simultaneous IC-RT-PCR and ELISA can be read by matching the IC-RT-PCR lanes a through f of tree 1 with ELISA bars 1 through 6 and lanes a through f of tree 2 with ELISA bars 7 through 12. Lanes – and + (negative and positive controls) are matched to ELISA bars 13 and 14 respectively. These results indicate that while CBI trees of C5 could show very mild symptoms on a few leaves the titer was very low. While, for example, only leaves in lanes 2 and 6 showed positive (although very low positive) ELISA values lanes c and d of tree 2 were ELISA negative but positive by the highly sensitive IC-RT-PCR technique.
a,b,c,d,e, f = six samples extracted from leaves from six different branches of a single tree.
B = B70146, untransformed trees; CBI = chip bud inoculation; AVI = aphid vectored infection.
- = negative control; + = positive control.

Accumulation of viral and transgene PPV-CP mRNA. RT-PCR confirmed in field samples the difference in transgene expression between PPV-susceptible transgenic clone

C3 and the resistant transgenic C5 clone (**Fig. 15**). C5 had little to no detectable transgene RNA, maintaining in the field a low level accumulation of transcript RNA, as had been shown in previous greenhouse studies (Scorza et al., 1994, 2001) demonstrating the stability of the PTGS mechanism in the field.

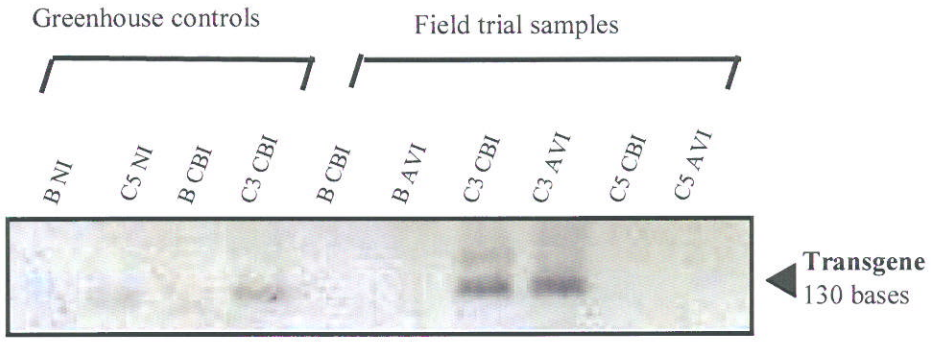

Figure 15. Detection by RT-PCR of transgene RNA and viral RNA accumulation. RT-PCR analysis of transgene RNA accumulation in plum leaves. C5 had little to no detectable transgene RNA, maintaining in the field a low level accumulation of transcript RNA, as had been shown in previous greenhouse studies (Scorza et al., 1994, 2001) demonstrating the stability of the PTGS mechanism in the field. Position and expected size of the transgene mRNA is indicated. [see Ravelonandro et al. (1992)].B = B70146, untransformed trees; NI = non-inoculated; CBI = chip bud inoculation; AVI = aphid vectored infection.

In summary, the Poland field test demonstrated that C5 was highly resistant to PPV. No plants were infected by aphids. C5 trees infected artificially by chip budding or via infected susceptible rootstock showed only a few mild symptoms on single isolated shoots. In the majority of leaf samples from these trees the virus was undetectable by ELISA and RT-PCR up to 8 years post-inoculation (Malinowski et al., 2006). These results clearly indicate the long-term nature and high level of resistance of C5 to PPV.

Spain field test. One hundred plum trees were planted in April 1997 (Permission B/ES/96/16 by the Spanish Ministerio de Medio Ambiente) in an irrigated experimental plot located in Líria, Valencia, an area with a mild Mediterranean climate. Trees spaced at a distance of approximately 4 meters between trees and 5 meters between rows. Half of the total trees were transgenic clones (C4, C5, C6, PT-6 and PT-23), planted in 5-tree rows (two rows per clone). The rest of the trees were non-transgenic plums (B70146 on *P. marianna* and *P. salicina* 'Black Diamond' on *P. cerasifera*) planted between transgenic rows (**Fig. 16**). A total of 25 non-transformed plums were graft-inoculated with a PPV-D strain (3.3 RB/GF Mp15/GF; Asensio et al., 1999) by bark chips to provide inoculation for aphid transmission. This PPV isolate is defective in the epitope that recognize the monoclonal antibody 4BD7 (Cambra et al, 1994; López-Moya et al., 1994). The use of this PPV strain which could be distinguished from naturally occurring PPV strains allowed for the determination of resistance to additional PPV strains brought into the test orchard by aphids. Guard trees surrounding the plot were peach X almond hybrids which are sexually incompatible with the plums.

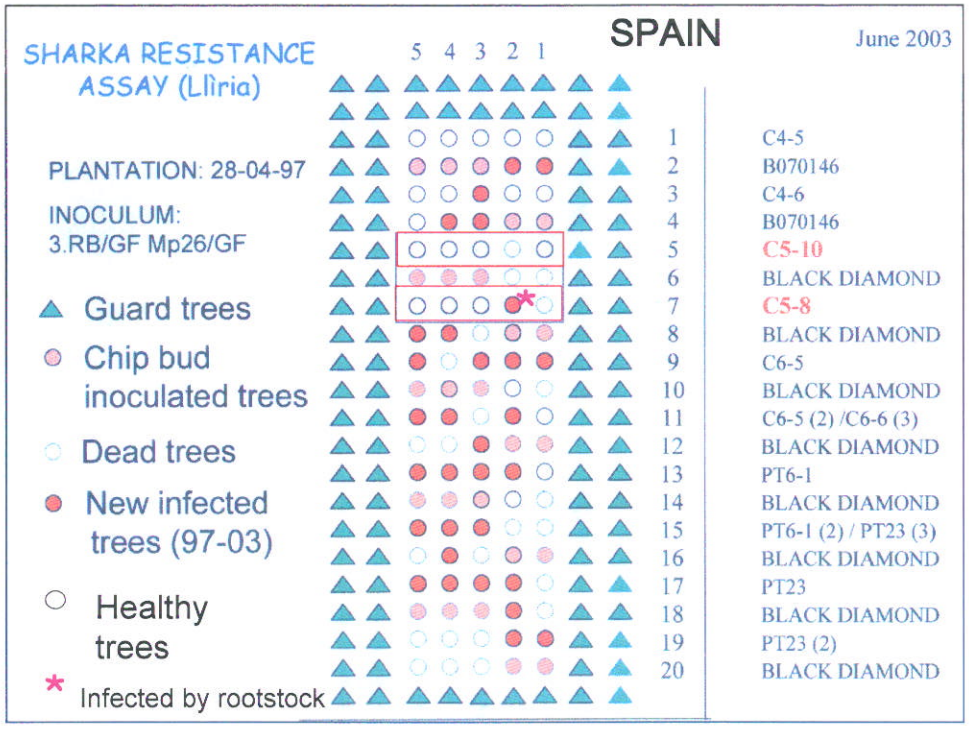

Figure 16. Field plot of PPV-CP transgenic plum field trial in Líria, Valencia, Spain. Transgenic clones C4, C5, C6, PT6, and PT23 and untransformed *Prunus domestica* B70146 were grafted on to *P. marianna* rootstocks. Untransformed *P. salicina* 'Black Diamond' was grafted onto *P. cerasifera*. Trees were field planted in 1997 and spaced at a distance of approximately 4 meters between trees and 5 meters between rows. 'Black Diamond' and B70146 trees were interplanted with all other clones in alternating rows and were chip-bud inoculated with PPV-D strain in the summer of 1997 to provide 25 inoculated trees that would provide inoculum for aphid transmission to transgenic trees. Guard trees surrounding the plot were peach X almond hybrids which are sexually incompatible with the plums. *Tree infected by rootstock indicates a C5 tree that was growing on a rootstock that was detected as infected presumably through aphid feeding on a shoot produced from a root. Shoot "suckers" from roots are commonly produced from *P. cerasifera*. Infection on this C5 tree was of a very low level and only detected on a few leaves adjacent to the graft union with the infected rootstock.

During the experimental period (1997-2003), the average of mean temperatures registered in the plot were 15 °C in spring, 24 °C in summer, 16 °C in autumn and 10 °C in winter, reaching a mean maximum of 31 °C in summer and a mean minimum of 3.5 °C in winter. The relative humidity oscillated between 58 and 68.5% in summer and autumn, respectively. The radiation was maximal in summer with an average value of 293 w/m^2 and minimal in winter reaching 101.7 w/m^2.

Natural aphid transmission. Total aphid individuals and the percentage of aphid species that visited the plot were determined in the spring of 2003 by the sticky shoot method (Avinent et al., 1993; Cambra et al., 2000).
The most common aphid species that visited the plot in Spain during the 2003 season were *Aphis spiraecola, A. gossypii* and *Hyalopterus pruni*. The temporal spread of PPV by aphids in the experimental plot is shown in **Fig. 17**. All C5 trees exposed to infection by viruliferous aphids (trees which were not chip bud-inoculated) remained uninfected by

PPV as evaluated by visual observation, ELISA, and RT-PCR. The other clone which showed relatively high resistance against aphid-vectored infection by PPV was C4. However, once a C4 tree became infected, it developed strong leaf symptoms which gradually spread throughout the entire tree. In contrast to the performance of C5, by the end of 2003 most trees of clones C4, C6, PT-6, PT-23, 'Black Diamond' and B70146 were infected. Two PPV serotypes were detected in the Spanish plot, including Mab 4BD7 negative serotype 3.3RB/GF Mp15/GF, which was introduced by inoculation (Asensio et al., 1999) and another serotype which likely came from the surrounding naturally infected plots. This serotype was Mab 4BD7 positive.

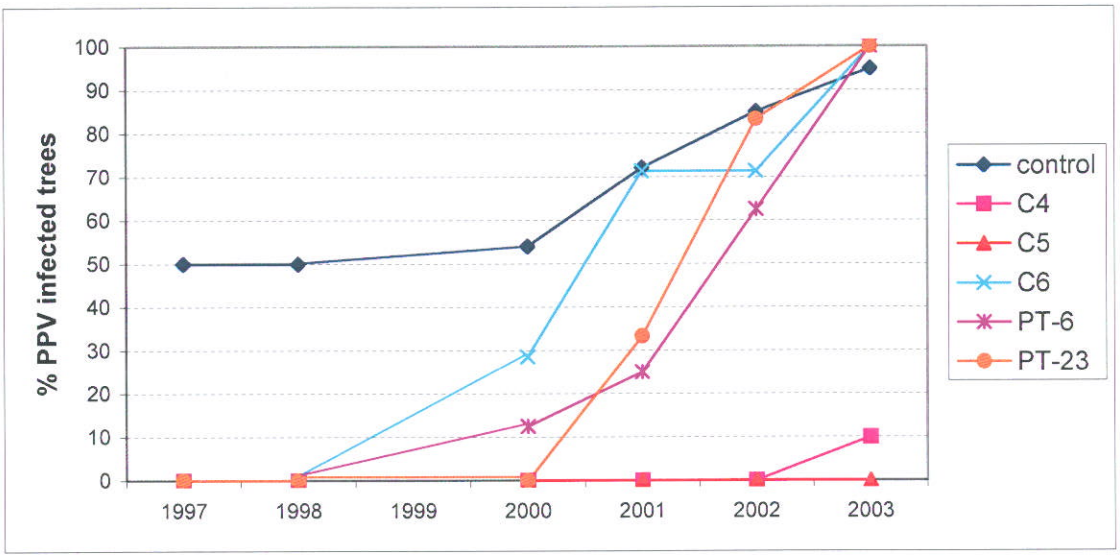

Figure 17. Temporal spread of PPV in control (non-transformed European plum 'B70146' and Japanese plum 'Black Diamond') and CP-PPV transgenic lines in the Spanish plot. Within seven years 100% of susceptible clones C6, PT-6 and PT-23, as well as nearly all non-transformed control trees were infected. Clone C4 exhibited a delay in infection remaining uninfected five years after inoculation. The first detection in C4 was in 2003, six years after orchard establishment, and only 1 out of 10 trees was infected. In 2004 (not shown), six out of ten C4 trees became infected, whereas the other four remained healthy as checked by visual observations, ELISA and IC-RT-PCR (**Fig. 2b**). C5 trees remained uninfected except for one tree which in 2004 showed a low level of infection close to the graft union from an infected rootstock.

In the case of one C5 tree in Spain, the rootstock was allowed to produce a branch comparable in size to the transgenic C5 scion. This rootstock was non-transgenic and became infected showing strong and clear symptoms. However, only one small shoot of C5 found in close proximity to the junction of rootstock/scion showed mild symptoms on few leaves and tested positively for PPV by RT-PCR and ELISA. Symptoms, positive ELISA, or positive molecular assays were not observed on other parts of the canopy of this C5 tree. The rest of the transgenic C5 plants, some of them on infected rootstocks remained uninfected (Malinowski et al., 2006).

MATERIALS AND METHODS FOR TREE EVALUATION IN SPAIN FIELD PLOT

Detailed observations of infection symptoms on leaves of all trees were carried out once a year and were accompanied by a collection of samples for laboratory tests. When collecting the samples from trees with suspected symptoms, four to six symptomatic leaves were picked from a tree for analysis.

In trees with no symptoms, leaves from 5-8 young shoots were collected around the canopy from the middle of each scaffold branch.

Samples were routinely tested for PPV by DAS-ELISA using DASI-ELISA withg the monoclonal antibody (Mab) 5B-IVIA (Cambra et al., 1994), or with a commercial ELISA kit (Durviz, Spain), according to standard protocols. Starting from 1999, Mabs 5B, 4DG5, 4DG11 and 4BD7 (Cambra et al., 1994), were used for PPV typing.

RT-PCR, IC-RT-PCR, RNA blotting, and biological indexing were used along with ELISA to confirm the presence of PPV infection. The same extracts as prepared for ELISA were used for RT-PCR or IC-RT-PCR. Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen) following manufacturer protocols. Primers 80 and 82 (provided by J. A. García, CNB, Madrid) amplified the C-terminal sequence of the NIb gene and the N-terminal sequence of the CP gene, (80-5'TTGGGTTCTTGAACAAGC3'; 82-5'TGGCACTGTAAAAGTTCC3'). Primers PPV-A and PPV-B (Koerschineck et al., 1991) amplified a fragment of the NIb cistron. Either set of primers could be used for specific detection of viral RNA by means of RT-PCR amplification. Primers PPV-A/PPV-B and P1/P2 (Wetzel et al., 1991) were used for IC-RT-PCR detection. The 490 bp fragments amplified with the 80 and 82 primers were sequenced and analyzed. Biological indexing was performed in a greenhouse, using the woody indicator GF305 and in a few cases, the herbaceous indicator *Chenopodium foetidum* (local lesion host for PPV). PPV RNA detection by RNA blotting was performed as described by Varveri et al. (1988) with minor modifications.

Evaluation of fruit infection. Serological (ELISA) and molecular (IC-RT-PCR) tests of fruit were conducted in Spain in 2003 on a sample of 3 fruit per tree from all C5 trees and 3 fruit per trees from 8 non-transformed trees. IC-RT-PCR reactions using P1 and P2 primers, that amplify the C-terminal of PPV CP gene, were negative for all the fruit samples, C5 and non-transformed. RT-PCR using 80 and 82 primers, that amplify the C-terminal of NIb gene and the N-terminal of CP gene from PPV, were also negative for all samples. In all cases, amplification was detected using a leaf extract from a PPV infected non-transgenic tree as positive control. In Poland, fruit analyses were not possible because flower buds were destroyed through 2002, a precaution undertaken to limit gene-flow *via* pollen from transgenic trees. No symptoms were noted on fruit produced on C5 trees in 2003. In Romania, over several years no symptoms have been observed on fruit of C5, while severe symptoms have been observed on susceptible transgenic and non-transformed trees.

Field test comparisons. Infection pressure was the highest in Poland likely due to a combination of numerous PPV infections in close proximity to test plants and high levels of aphid activity. Yet, in all three test sites, infection pressure was high enough to ensure the natural spread of PPV to nearly 100 percent of non-transgenic plums, providing a clear contrast to the performance of clone C5. The high level of PPV resistance of C5 against naturally vectored PPV infection was observed independently at three different locations with different strains of PPV present and under diverse climatic conditions.

Small quantitative difference appeared between results obtained for C5 trees inoculated by CBI in Poland and the C5 tree in Spain in which non-transgenic rootstocks became infected, apparently through aphid inoculation of root shoots (root suckers), which can be compared with a graft inoculation (CBI). In both field plots, some trees were exposed to a continuous supply of the virus, either from CBI in Poland, or infected

rootstocks in Poland and in Spain that had become infected presumably from aphid inoculation of root suckers. In Spain though, symptoms were milder with only one small symptomatic shoot very close to junction union. In Poland, the two CBI trees had more symptomatic leaves although considerably fewer than on susceptible trees. This difference was probably due to the higher temperatures in Spain. It seems that temperature at early spring may be important. The symptoms observed on CBI trees in Poland were extremely few and mild in 2000 and 2003 – two years with the highest temperature records during the spring. Similarly, PPV was not detected in the first experiments performed in the glasshouse with graft-inoculated C5 (Ravelonandro et al., 1997). Later, it was reported that PPV could be sometimes detected in the inoculated C5 grown in the glasshouse by using IC-RT-PCR (Scorza et al., 2001), especially in the beginning of the season. It seems that PTGS mechanism may not be functioning with full efficiency immediately following the winter period.

Four major isolates of PPV have been previously characterized that differ in their biological, serological and molecular properties: PPV-D (Dideron), PPV-M (Marcus), PPV-C (that include the closely related isolates PPV-SoC and PPV-SwC) and PPV-EA (Kerlan and Dunez, 1976; Wetzel et al., 1991; Nemchinov and Hadidi, 1996; Nemchinov et al., 1996). In Spain and Poland, only the strain D of PPV, that affects mainly apricot and plum trees, is presently spreading. Both PPV-D and PPV-M are found in Romania. In our experiments different sub-types of D were identified in plants grown in Poland, Spain, and Romania, with PPV-M also found in the Romania test plot (Ravelonandro et al. 2002b). This provides additional support for the evaluation of C5 clone as highly resistant to the major serotypes of PPV (Ravelonandro et al., 2001) and its resistance as durable.

These challenging assays demonstrated the resistance of clone C5 to PPV under high infection pressure and under a wide range of environmental conditions, virus isolates and aphid vectors for seven years in the field, a period of time representing a significant part of the productive life of a plum orchard.

Resistance to multiple strains of PPV. (from Ravelonandro et al., 2001a; and unpublished data). The objective of these studies was to observe the behavior of the transgenic clone C5 when challenged with the four major serotypes of PPV and to understand the gene-for-gene correspondence that we speculate may be responsible for protection. The study was carried out under containment conditions, under permit, at INRA, Bordeaux, France.

Transgenic plums utilized in these studies were derived from plum transformation of hypocotyl slices with *Agrobacterium tumefaciens* as described by Scorza et al. (1994). Two clones were selected, C5 and C6, neither of which produce transgene CP. C6 contains one copy of the PPV-CP insert and unlike C5, is PPV susceptible (Scorza et al.,1994 ; Ravelonandro et al, 1997). The C5 and C6 mother plants were grown in large pots in the containment greenhouse. To produce the required plant materials for the study, buds from transgenics were grafted onto peach GF-305 rootstocks that had been previously infected with the four PPV serotypes. Plants were then grown in a greenhouse maintained at 22-24°C.

Four PPV strains from different PPV serotypes, D, M, El Amar and Sour Cherry, were selected for challenging. PPV infection was monitored by symptom appearance at 5

weeks following bud break. The four different PPV serotypes were derived from different *Prunus* species: PPV D from *P. domestica*, M from *P. persica*, El Amar from P. armeniaca and Sour Cherry from *P. avium*. While infections caused by these different serotypes cannot be readily distinguished by symptoms, the CP of these respective serotypes show a different global charge, which can be confirmed by the different electrophoretic mobility of each protein when fractionated on denaturing polyacrylamide gel. Leaves were collected for DAS-ELISA and western-blotting experiments for the detection of PPV-CP (Ravelonandro et al, 1997).

To evaluate PPV infection in the tested scions, both non-transformed rootstocks and transgenic shoots were monitored. Visual symptoms began to appear in the GF-305 rootstocks and in the susceptible C6 clone five weeks following the growth flush. After four months of growth, scions were moved into a cold chamber for inducing artificial dormancy where they remained for three months (Ravelonandro et al, 1997). Following dormancy, a high incidence of PPV was observed in control plants.

Because the two transgenic clones do not express CP, infection monitoring was based on the detection of CP. Three to five transgenic plums were generated for each PPV serotype inoculation and evaluated for PPV infection. All tested roostocks showed systemic symptoms and positive DAS-ELISA. Prior to dormancy, the majority of C6 plants were infected, but none of the C5 plants were infected. Following dormancy, all C6 scions were infected. The analysis of PPV contained in young leaves showed that protection occurred in clone C5. A band of about 36-38Kd, corresponding to CP, was observed in the different samples from both rootstocks and transgenic C6, however, there was no viral protein detected in the transgenic plum C5 (not shown). Clone C5 showed no infection when plants were tested for D, M, El Amar or Sour Cherry strains. These results show that the protection is broad over the major PPV serotypes.

Resistance to Prunus viruses other than PPV. Evidence from inoculation trials conduced under greenhouse conditions at INRA Bordeaux, France and under field conditions at the Research Institute of Crop Production (Czech Ministry of Environment permit 881/OER/GMO/01) indicate to date that the C5 clone is susceptible to Prunus necrotic ringspot and apple chlorotic leafspot viruses. Inoculations with other common *Prunus* viruses are underway.

XI. Inheritance of Transgene and Plum Pox Virus Resistance in the Progeny. [*This section presents transgene inheritance and in those studies where hybrid seedlings were inoculated with PPV, the results of these inoculations are presented.*]

Intraspecific hybridization between C5 and Papaya ringspot virus (PRV) coat protein (CP) transgenic P. domestica and resistance of seedlings to PPV (Scorza et al., 1998). C5 plants were transferred from tissue culture conditions to a greenhouse in Feb 1993 and grown in the greenhouse at 27°C for 27 months under a mixture of natural and supplemental lighting. After 11 and 23 months of growth (Jan 1994 and Jan 1995), plants were placed at 4°C in the dark for 60 days and then returned to the greenhouse. Plants flowered at month 27 (April 1995). Pollen was collected and stored at 4°C for approximately one week before use.

Transgenic plum trees carrying the papaya ringspot virus (PRV) CP gene, located at the USDA-ARS Appalachian Fruit Research Station, Kearneysville, WV, were described in Scorza et al. (1995). These trees were field planted under U.S. Animal Plant Health Inspection Service (APHIS) field test permit 92-191-01 in Nov 1992. At flowering, trees were covered by boxes made of construction sheathing to eliminate natural cross-pollination. Flowers on two or three trees of PRV-CP transgenic clones EF1 and EF2 respectively were hand-emasculated one to two days prior to anthesis by pinching-off sepals, petals, and stamens. Pollen of C5 was applied to emasculated flowers. Additional EF1 and EF2 trees were covered to allow for self pollination. Trees remained covered until flowers were non-viable as indicated by the cessation of pollen shedding and the desiccation of styles.

Fruits that developed from cross pollination were individually tagged and harvested at a green-ripe stage. Seeds were excised from the stones and stratified at 4°C for 70 days. Following stratification, seeds were planted in a greenhouse under the conditions described above. Inheritance of the PRV and PPV-CP transgenes was confirmed by polymerase chain reaction (PCR) using primers for both the PRV- and PPV-CP genes as described in Scorza et al. (1994, 1995) and using similar cycle parameters.

Seedlings were pruned when approximately 25 cm tall. The pruned shoots supplied budwood for the propagation of each seedling. Chip buds from each seedling were inserted into Myrobalan plum (*Prunus cerasifera*) rootstocks and at the same time, under containment conditions at ARS, Ft. Detrick, a bud from a PPV-infected *P. tomentosa* (PPV isolate described in Scorza et al., 1995) was also inserted into each rootstock at a point basal to the transgenic seedling bud. Control plants for inoculation tests were uninoculated plants of each transgenic seedling and inoculated and uninoculated plants of the parental clones C5 (PPV-CP transgenic), EF1 (PRV-CP transgenic), EF2 (PRV-CP transgenic) and inoculated and uninoculated untransformed plums 'Bluebyrd' (formerly selection B69158) and selection B70146 which were the original seed sources for PRV- and PPV-CP transformation experiments. The transgenic and inoculum buds were allowed to grow. Following observation of shoot growth from the inoculum buds to verify symptom expression on the inoculum, these shoots were eliminated leaving only the transgenic shoot to grow from the rootstock. PPV infection was evaluated through ELISA, and by observation of symptom expression.

From a total of 32 seeds obtained from cross hybridization between C5 and EF1 and EF2. Twenty-seven seed appeared normal and five were shriveled and non-viable. Of the 27 seeds stratified, seven plants were obtained. A total of 13 seedlings were obtained from self-pollination of EF1 and EF2 trees. The low production of fruit and viable seeds from these hybridizations may be attributed, at least in part, to high ambient temperatures during pollination. Temperatures outside the enclosures reached as high as 35 °C during this time. It is assumed that temperatures inside the enclosures were even higher. The negative effects of high temperatures on fruit set have been discussed by Layne (1983). PCR analysis using PRV-CP and PPV-CP primers showed that of the seven hybrids, including two that were obtained from open pollination of EF2 (lanes F and G **Fig. 18**), three contained both the PRV-CP and PPV-CP genes and two contained only the PPV-CP gene from the C5 pollen parent (**Fig. 18**). Self-pollination of EF1 and EF2 produced 10 of 13 seedlings with the PRV-CP transgene. Evaluation of the

transgene positive controls revealed that the PRV-CP transgenic parent trees EF1 and EF2 contained only the PRV-CP gene, and the C5 PPV-CP transgenic parent contained only the PPV-CP gene, as expected.

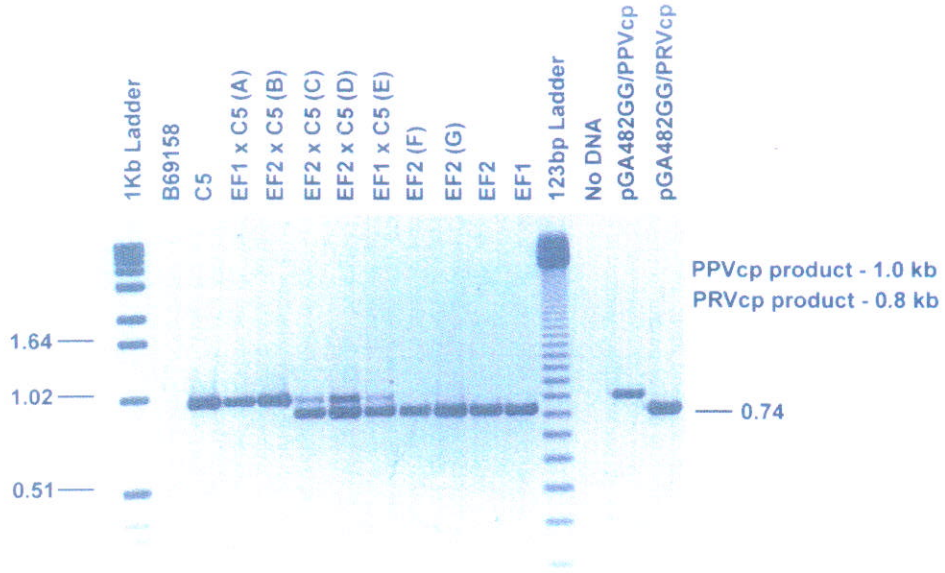

Figure 18. PCR analysis of the plum pox virus (PPV) and papaya ringspot virus (PRV) coat protein (CP) genes in hybrids between PPV-CP transgenic clone C5 and PRV-CP transgenic clones EF1 and EF2 (lanes A, B, C, D, E). Lane A is clone 1 on Table 5; lane B clone 11; lane C clone 5; lane D clone 6; lane E clone 10. Lanes F and G (clones 8 and 15) are progeny from self-pollination of EF2 and EF1, respectively. B69158 is a nontransgenic control; C5, EF1, EF2 are parental trees, the last two lanes represent plasmid DNA containing either the PPV or PRV gene.

At eleven months, post-inoculation PPV symptoms were observed on inoculated control plants and on transgenic plants containing only the PRV-CP gene. ELISA data presented as a total of plants infected for all evaluation dates showed that non-transgenic control and PRV-CP transgenic plants were ELISA positive indicating that they were infected with PPV. Untransformed control plants of clone 70146 were ELISA negative. This was possibly due to their poor growth and the lack of leaf material most suitable for ELISA. The C5 parent and C5 progeny containing the PPV-CP genes inherited from the C5 parent were ELISA negative (**Table 5**).

Table 5. Evaluation of transgenic hybrid plum clone resistance to PPV infection. ELISA data presented as a total of plants infected at eleven months post-inoculation. Non-transgenic control and PRV-CP transgenic plants were ELISA positive indicating that they were infected with PPV. Untransformed control plants of clone 70146 were ELISA negative. This was possibly due to their poor growth and the lack of leaf material most suitable for ELISA. The C5 parent and C5 progeny containing the PPV-CP genes inherited from the C5 parent were ELISA negative

Clone I.D.	Inherited Genes [a]	Symptom Expression	ELISA Results [b]
1	PPV-CP [c]	-	- (5/5) [e]
5	PPV-CP, PRV-CP [d]	-	- (7/7)
6	PPV-CP, PRV-CP	-	- (9/9)
8	PRV-CP	-	- (4/5), +(1/5)
10	PPV-CP, PRV-CP	-	- (6/6)
11	PPV-CP	-	- (6/6)
15	PRV-CP	+	- (1/5), +(4/5)
C5 [f]	PPV-CP	-	- (7/7)
EF1 [f]	PRV-CP	+	- (2/3), +(1/3)
EF2 [f]	PRV-CP	+	- (4/8), +(4/8)
69158	untransformed control	+	- (0/4), +(4/4)
70146	untransformed control	-	- (2/2)

[a] Inheritance of transgenes determined by PCR analysis (see **Fig**. 1).
[b] ELISA results of PPV inoculated plants. Uninoculated plants were all ELISA negative. ELISA was performed using polyclonal antisera to the PPV coat protein. Transformed uninoculated controls produced levels of coat protein in quantities below the limit of detection in our ELISA test. Therefore, the PPV-CP ELISA was used for these preliminary tests.
[c] PPV-CP is the plum pox virus coat protein gene.
[d] PRV-CP is the papaya ringspot virus coat protein gene.
[e] (-) indicates ELISA negative (+) indicates ELISA positive. Numerator is the number of plants ELISA positive or negative, denominator is the total number of plants tested for the particular transgenic or control clone. The number of ELISA positive plants are combined over two sampling dates.
[f] Parental clones.

Intraspecific hybridization between C5 and commercial French P. domestica cultivars and resistance of hybrid progeny to PPV (Ravelonandro et al. 2002a). This work carried out in a containment greenhouse at INRA, Bordeaux, France. C5 was used as the male (pollen) parent and 'Prunier d'Ente 303' and 'Quetsche 2906' as females. All hybridizations were performed on potted plants enclosed in an insect-proof screen cage grown in the containment greenhouse. Prior to full bloom, flowers of 'Prunier d'Ente' and 'Quetsche' were emasculted by removing anthers. Pollen of clone C5 that had been previously collected and air-dried was placed on the stigmas of emasculated flowers of 'Prunier d'Ente' and 'Quetsche'. Fruits were harvested after maturation and seeds collected. Seeds were germinated in KNOP medium with 2% sucrose before transfer into soil and greenhouse conditions.

To evaluate the transfer and expression of the GUS transgene marker, leaves of each putative hybrid were collected and independently treated with X-glucuronide substrate (Jefferson, 1987). GUS assays were repeated twice for each putative hybrid. DNA was extracted from a sample of leaves of each putative hybrid using the Dneasy-

QIAGEN kit (QIAGEN Inc. Chatsworth, CA). PCR detection of the transgene PPV-CP cistron followed the methods of Scorza et al. (1994).

A sample of seedling clones was selected for PPV inoculation studies. Two inoculation techniques were utilized. For in vitro evaluation of infection, transgenic and control shoots were maintained in Murashige and Skoog (MS) medium supplemented with giberrellic acid (GA3) and 2% sucrose. GF305 peach (*P. persica*) roostocks pre-inoculated with PPV were grown on the same medium in vitro. Five to 10 replicates of each selected transgenic and non-transgenic hybrid grown in vitro were micrografted onto the PPV-infected GF305 rootstock. When the micrografted scions grew for three to four weeks, samples were collected from the bottom and the top part of the scions for monitoring virus spread. Plants of these same seedling clones were grown in the greenhouse and tested by chip bud inoculation of PPV from infected GF305. PPV infection was evaluated either by immunoblot detection using the PPV-CP specific antiserum, because the C-5 transgenic parental clone does not express PPV-CP, or by IC/PCR using PPV-CP specific primers developed by Wetzel et al, 1992 or Korschineck et al, 1991.

Positive GUS assays were obtained for 40% of 'Quetsche' x C5 hybrids (16 of 40 seedlings) and 49% (56 of 115 seedlings) of 'Prunier d'Ente' x C-5 hybrids. PCR assays confirmed positive GUS assays in all cases. The ratios of transgenic to non-transgenic seedlings suggest a single gene Mendelian pattern of inheritance of the inserted transgene cassette with the parental C5 heterozygous for the transgene and the transgene allele absent in the 'Prunier d'Ente' and 'Quetsche' parents, producing a 1:1 ratio of transgenic to non-transgenic hybrids (Table 6).

Analysis of micrografts showed that PPV was detected in all preinoculated rootstocks and in the non-transformed shoots, however no virus was found in transgenic shoots (Fig. 19). Immunodetection analysis of plants confirmed these results. DAS-ELISA OD readings were relatively high in GF8-1 rootstocks and non-transformed hybrids when compared with transgenics that remained PPV negative, including the C-5 clone male parent (not shown). Transgenic hybrid clones were consistently negative for PPV infection by symptomology and by immunodetection following two dormancy cycles (each cycle 2 months at 4° C). These results support the previous observations with (PRV-CP x PPV-CP) hybrid plums and indicate that the transgene cassette introduced *via* cross-hybridization with C5 can be regarded as stably inherited and functional in hybrid seedlings. The Mendelian inheritance as a single gene (locus) dominant trait was indicated.

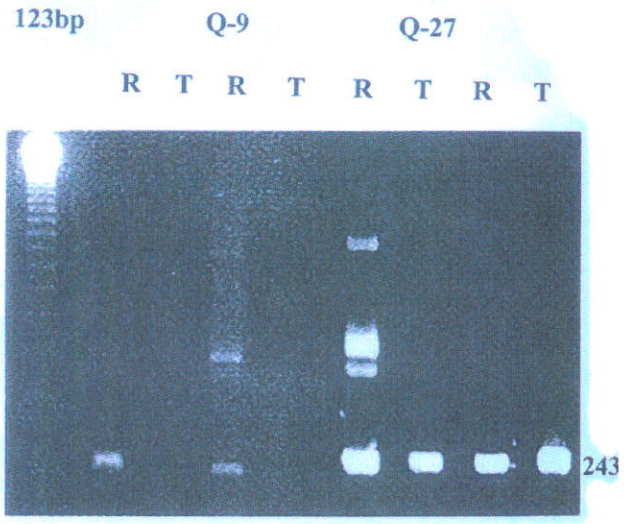

Figure 19. Electrophoresis in an agarose gel of IC/RT-PCR products derived from 'Quetsche' x C5 hybrids (transgenic clone Q-9 and non-transformed Q-27) at 4 weeks after micrografting on PPV –infected GF-305 peach rootstocks. R=rootstock and T=tested shoots.

Open-pollination of C5. Under a 2002 amendment to APHIS permits 92-191-01 and 95-205-02 transgenic plum trees (*P. domestica*) were allowed to open-pollinate in field plantings at USDA-ARS AFRS, Kearneysville, WV. Fruit were collected from trees of C5. Seed were extracted, stratified and germinated as above. The GUS histological assay and DNA blotting utilizing a PPV-CP specific probe were used to evaluate inheritance of transgenes and specifically transfer of the unique multicopy C5 PPV-CP insert (**Fig. 20**). In all cases studied to date the banding pattern of transgenic seedlings matches the pattern of the C5 parent indicating that the multicopy PPV-CP insert is stably inherited as a single locus.

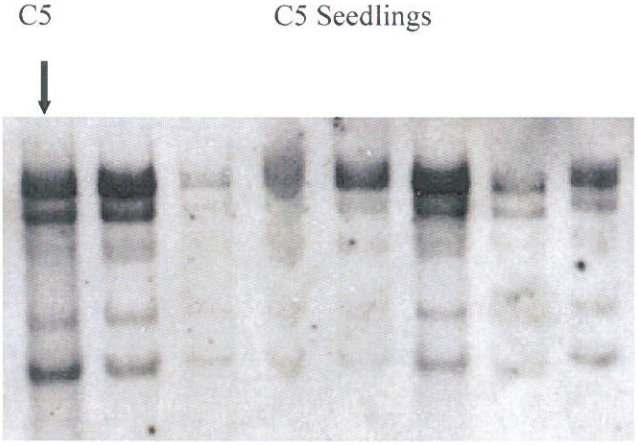

Figure 20. DNA blot (*Eco*RI digest) of seedlings of open-pollination of C5. The PPV-CP probe was generated through PCR using primers and amplification parameters as specified in Scorza et al. (1994). The C5 parent, which was the female source of the seedlings, is shown in the far left lane. The banding pattern of transgenic seedlings matches the pattern of the C5 parent indicating that the multicopy PPV-CP insert is stably inherited as a single locus.

Over a period of several years seedlings from open-pollinated C5 at the Kearneysville field planting have been evaluated for the inheritance of the transgene insert (**Table 6**). These studies, based upon the evaluation of GUS through a histological assay (Jefferson, 1987) demonstrated a1:1 segregation for transgene presence: absence indicating a Mendelian segregation of the transgene locus. To confirm the correlation between presence of the GUS and PPV-CP genes in seedlings, 50 seedlings from C5 open-pollination II (**Table 6**) were evaluated through DNA blotting as described in section VII *Molecular Characterization of transgenic clone*. Of the 50 GUS positive C5 seedlings tested, 49 produced the C5 banding pattern typical of *Eco*RI digestion. One seedling contained the PPV-CP transgene but the banding pattern was not typical for C5 and could have been the result of cross-pollination between C5 and another PPV-CP transgenic clone in the planting. (The hybrid nature of this seedling could not be confirmed and further observations have not revealed such hybrids.) The results of open pollination of C5 confirm results of controlled hybridizations with C5 which show a strong linkage between the GUS and PPV-CP gene(s).

Interspecific hybridization between P. spinosa and C5 and PPV resistance of seedlings (Ravelonandro et al., 2001b). [This work was carried out under containment conditions at INRA, Bordeaux, France.] Pollen from the C5 donor was collected and then dried overnight. The *P. spinosa* clone used as the female parent was known to be interfertile with *P. domestica* (M. Ravelonandro, pers. comm.). *P. spinosa* can range in ploidy level from diploid to hexaploid, depending on the clone (Section II. *The Genus Prunus. Number of chromosomes*). The *P. spinosa* clone used in this study was a tetraploid. Flowers of *P. spinosa* were emasculated and pollen of C5 was applied on the stigmas. Fruits were harvested and seeds were grown on Murashige and Skoog (1962) culture medium. *In vitro*-grown seedlings were transferred into soil and acclimatized during two months in a growth chamber with a photoperiod of 16h light and 8h dark 24 °C.

To detect transgene integration into the hybrid plants, expression of different transgenes carried by the T-DNA was assayed. Histological tests for *uidA* (GUS) expression were performed (Jefferson, 1987) and GUS-positive clones were subjected to PCR following the procedures of Scorza et al. (1994).

PPV strain M (from Greece) was selected for inoculation based on its rapid spread in a young *Prunus* test plant. Scions of transgenic seedlings were grafted onto GF8-1 rootstock and these shoots and non-transformed controls were chip-bud inoculated as described by Ravelonandro et al. (1997). Infection was monitored by symptom appearance at 6 weeks following the bud break when leaves were collected for DAS-ELISA testing (Ravelonandro et al, 1997). These assays were repeatedly conducted during the growth flush. Total plant proteins were extracted and then analyzed by immuno-blotting for the detection of PPV CP or the helper component protein (HC) (Ravelonandro et al, 1997, 1998).

Following the cross-hybridization, 97 seedlings were produced. Forty-four seedlings were GUS-positive. Repeated testing of GUS-negative plants did not reveal additional positives. These results show the sexual compatibility between *P. domestica* transgenic clone C5 and *P. spinosa.* Further, the GUS assays show the frequency of transgenic hybrids was about 45%. This ratio follows a pattern of Mendelian single gene

segregation and again confirms that the multiple transgene inserts of C5 are closely linked and are transmitted as a single dominant gene (locus) (Scorza et al., 1998).

PCR detection of both NPTII and PPV CP cistrons using the respective oligonucleotides confirmed the results of GUS histological assays and indicated that the PPV CP and NPTII genes were also integrated into the transgenic seedlings (see Ravelonandro et al 2001).

Immuno-blotting showed a band of 36Kd, corresponding to PPV CP, was observed in the different sampled areas of the non-transformed control scion (S-94), however, there was no viral protein detected in the transgenic hybrid THS-95 demonstrating that the PPV CP transgene-based resistance effectively functions when transferred through interspecific hybridization (**Fig. 21**).

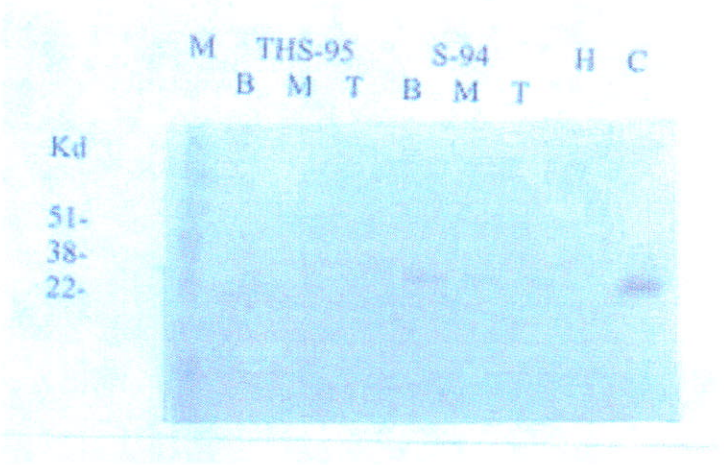

Figure 21. Immuno-blot assay of total protein extracts reacting with polyclonal antibodies raised to PPV CP. Total proteins were obtained from leaves located at three positions in the grafted shoots, bottom (B), middle (M), and top (T) of the transgenic hybrids THS-95 (*P. spinosa* x C5) and the non-transformed hybrid S-94. Lane M represents the benchmark prestained protein markers, lane H is a healthy control, and C is an infected control . PPV-CP was only visualized in the infected control (C) and in lanes B and M of S-94, the untransformed infected plum (Ravelonandro et al., 2001).

Summary of the inheritance of the C5 transgene insert. Considering controlled hybridizations in the U.S. and France and open-pollination in the U.S., the segregation of the transgene insert indicates transmission in a Mendelian fashion as a single gene (locus) dominant trait (**Table 6**). It should also be noted that the parent clone continues to express both the *nptII* and GUS genes, and these genes are also transmitted to the progeny as functional components of the C5 transgene insert (data not presented). Based on the inheritance data obtained to date, the components of the C5 insert, identified by restriction digests and DNA blotting, appear to be located at one insertion site in the C5 genome.

Table 6. Inheritance of the C5 transgene insert based on GUS assays of seedlings.

Cross	Total seedlings	GUS+	Segregation	χ square	P
C5 x 'Prunier d'Ente'	115	56	1:1	0.035	0.85
C5 x 'Quetsche'	40	16	1:1	1.23	0.25
C5 x *P. spinosa*	97	45	1:1	0.503	0.48
C5 open pollinated pop. I	218	118	1:1	1.48	0.27
C5 open pollinated pop. II	212	120	1:1	3.68	0.06

PPV-CP transgene methylation and siRNA in C5 progeny. The transfer of the transene and PPV resistance to progeny of C5 is clearly demonstrated. While the mechanism of resistance in the parental line is apparently PTGS-based, the following work evaluates PTGS in progeny of C5 and temporal aspects of processes involved in PTGS in the seed and seedlings. In herbaceous systems, PTGS has been shown to be "re-set" following seed germination, generally being reinitiated upon virus infection. To our knowledge, this phenomenon has not been studied in woody perennials. *P. domestica* normally requires stratification (moist chilling) for germination. Seeds of open-pollinated C5 carrying the PPV-CP insert were germinated with and without stratification, as were seeds of C3, a non-PTGS PPV-CP transgenic clone that is susceptible to PPV. Seeds or seedlings used in this study were not inoculated with PPV. DNA blots from samples of 1 month post-germination transgenic seedlings restriction digested with methylation-sensitive *Sau*3AI and methylation insensitive *Mbo*I indicated that the PPV-CP in these young seedlings was specifically methylated. The *uid*A gene in these same seedlings of C5 did not show evidence of methylation. DNA from leaves of C3 seedlings and parent appeared to be unmethylated in both the PPV-CP and *uid*A genes (**Fig. 22**). Quantitative PCR indicated reduced methylation prior to germination in C5 derived embryos and seedlings 2 weeks post-germination, but levels returned to those in C5 parental leaves one month after germination. DNA from seedling and parental leaves appeared to be more methylated at position 340 than at position 660 of the PPV-CP transgene. C3 displayed low methylation levels at both sites in all samples analyzed (**Fig. 23**). C5 embryos and 2-week post-germination samples displayed increased levels of PPV-CP transcript, whereas, leaves from 1 month old seedlings expressed relatively low levels like the leaves from the parental tree. These data indicated that in the embryo and at the early stages of germination and seedling development (prior to 1-month) PTGS is not fully functioning. By 1-month the process is fully functional being at the level of the mature C5 clone. In contrast, the C3 samples displayed relatively high levels of PPV-CP mRNA throughout development verifying that this clone and its seedlings to not display PTGS (**Fig. 24**).

siRNA was detected in embryos from ungerminated seeds and from 2-week and 4-week post germination samples, as well as from samples of leaves from mature C5 trees (**Fig. 25**).

These results suggest that although the expression of PTGS was altered in seeds and in seedlings of C5 two weeks post germination, at one month post germination all components of the PTGS mechanism that were evaluated, including siRNA production, were at levels comparable to those found in leaves of the parental C5 clone. These

results demonstrate the transfer of all components of the PTGS mechanism to seedlings of C5. The early PTGS activity, even in the absence of PPV inoculation, further suggests the value of C5 as a parent in breeding for resistance to PPV.

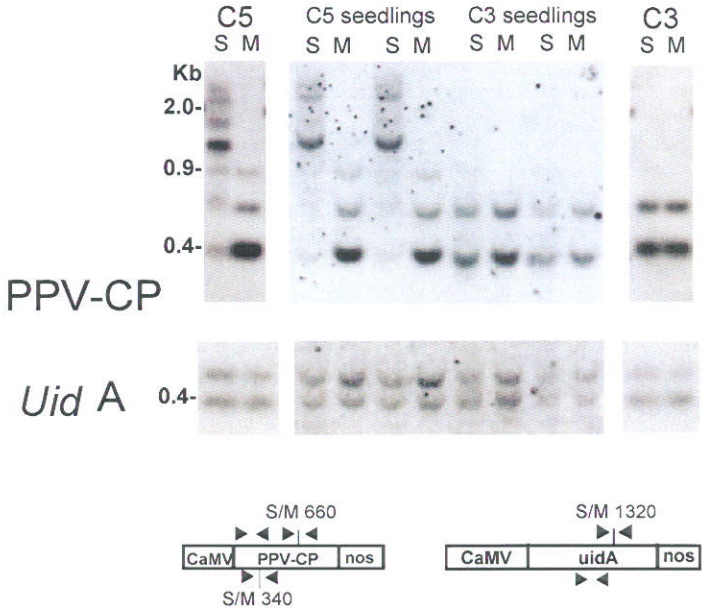

Figure 22. DNA blots from samples of leaves from one month post-germination transgenic seedlings from transgenic clones C5 and C3 restriction digested with methylation-sensitive *Sau*3AI and methylation insensitive *Mbo*I. C5 and C3 parental plants (left and right, respectively) are controls. These digests show that the PPV-CP gene is methylated because *Sau*3AI did not cut to completion (compare with *Mbo*I). The *uid*A gene in these same seedlings of C5 did not show evidence of methylation indicating the PPV-CP gene in C5 is specifically methylated. DNA from leaves of C3 seedlings and parent appeared to be unmethylated in both the PPV-CP and *uid*A genes. S = methylation sensitive *Sau*3AI, M = methylation insensitive *mbo*I. Schematic diagrams of PPV-CP and *uid*A with primer locations for Quantitative PCR and RT-PCR assays.

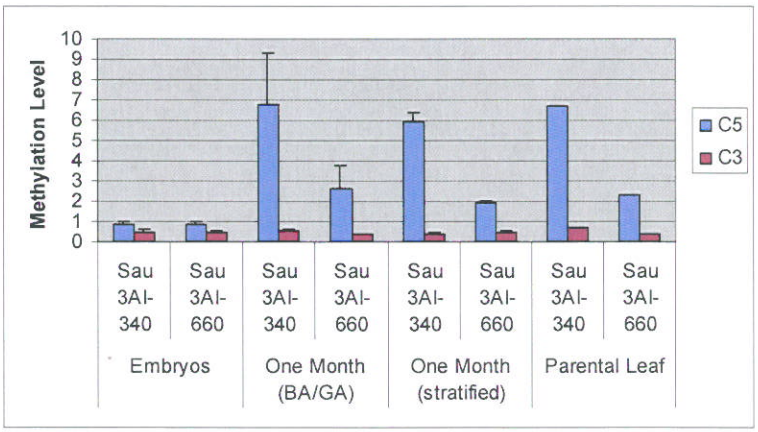

Figure 23. Relative methylation quantification by PCR (TaqMan). The x-axis indicates the different developmental stages evaluated for methylation: sites 340 and 660 were methylation sensitive site (Sau3AI) in the PPV-CP sequence. Values represent levels of amplification obtained from the threshold cycle number. There is a linear relationship between the amplification value and the amount of amplifiable

starting material. The greater the level of DNA methylation, the higher the amplification value. Values are the average of at least three independent experiments using samples of ten embryos and seedlings per experiment. Three to five mature plants were analyzed per clone.

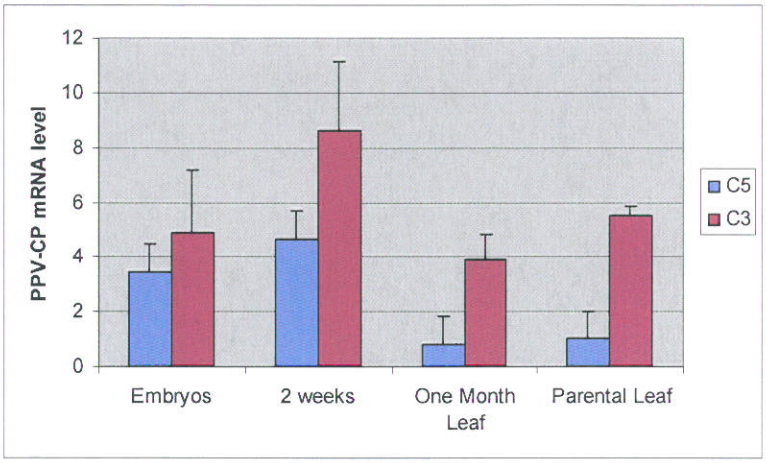

Figure 24. Relative mRNA quantification by RT-PCR (TaqMan). The x-axis indicates the different developmental stages evaluated. Values represent levels of amplification obtained from the threshold cycle number. There is a linear relationship between the amplification value and the amount of amplifiable starting material. The greater the level of RNA expression, the higher the amplification value. Values are the average of at least three independent experiments using ten embryos per experiment and 3-5 parental plants per line.

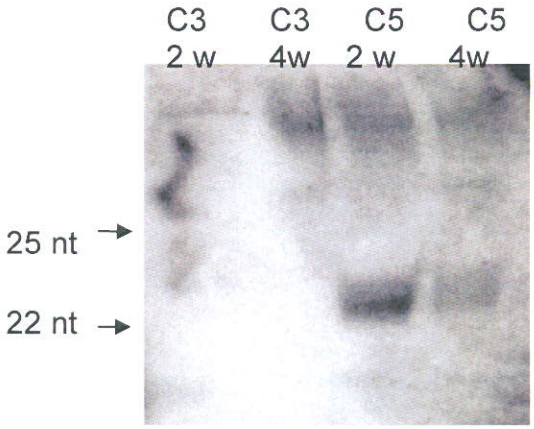

Figure 25. Phosphor-Image showing siRNA detection in C5 total RNA extracts from uninoculated seedlings of transgenic clones C3 and C5 at 2 and 4 weeks post germination. siRNAs are only present in C5.

MATERIALS AND METHODS FOR METHYLATION AND siRNA ANALYSES

Methylation analyses. Non-PPV-inoculated progeny of hybridizations between clone C5 and susceptible plum lines (Scorza et al., 1998) were assayed for methylation by hybridizing restriction digests (double digests of *Bam*HI and MboI or *Bam*HI and *Sau*3a) of C5 and C3 DNA with the PPV-CP probe previously described (Scorza et al., 2001). For quantitative PCR, primers were designed to flank the *Sau*3AI restriction sites at positions 340 and 660 in the coding region of the PPV-CP. Increased amplification from *Sau*3AI digested

DNA with these primers correlates with an increase in methylation at the restriction sites. Control primers were selected within this sequence that did not contain a *Sau*3AI restriction site. PCR reactions utilized SYBR® Green PCR Master Mix and data was normalized to amplification resulting from primers in the unmethylated *uid*A gene using the Comparative Ct method (Applied Biosystems).

RNA analyses. Total cellular RNA was isolated using a PureScript RNA isolation kit (Gentra Systems, Inc.). The lysis buffer of this kit was amended with 2% w/v PVP-40 per the manufacturer's recommendation for woody plant isolations. Contaminating DNA was removed from these samples using a DNA-Free kit (Ambion). RT-PCR reactions utilized Taqman® One-Step RT-PCR Master Mix Reagents (Applied Biosystems). The control primers employed for DNA methylation analyses were used as gene specific RT-PCR primers for the PPV-CP and *uid*A genes. siRNA was detected using P32-labeled PPV-CP probes following denaturing PAGE and electro-blotting to Hybond-NX membranes (Amersham Biosciences)

XII. Gene Flow From Transgenic Plum Trees

Two adjacent and contiguous plantings of transgenic plums are located on property of the USDA-ARS Appalachian Fruit Research Station, Kearneysville, WV. The total area of both blocks is approximately 1.5 acres (**Fig. 26 and 27**). Transgenic trees containing the papaya ringspot virus (PRV) coat protein (CP) gene, NPTII and *uidA* (GUS) marker genes were planted under permit APHIS 92-191-01. Currently, three transgenic trees remain in this block. Under permit 95-205-02 sixty-six transgenic trees were planted containing the plum pox virus (PPV) CP gene, NPTII and GUS marker genes including nine own-rooted trees of C5 that had been propagated through tissue culture from the original transgenic clone and confirmed for the C5 insert pattern through restriction digests and DNA blotting. Under permit amendments, pollen movement has been monitored within the PPV-CP block and between the PPV-CP and PRV-CP blocks and non-transformed *P. domestica* blocks at the Appalachian Fruit Research Station.

Intra-experimental block pollen flow. In 2003, 50 seeds were collected from one tree each of untransformed *P. domestica* selection B70146 and 'Bluebyrd' (formerly selection B69158) growing in the PPV-CP transgenic block. The embryos of these seeds were analyzed for GUS expression through the histological assay (Jefferson, 1987). None of the embryos were GUS positive (positive controls confirmed the assay). The apparent lack of pollen transfer within this block of trees may be due to an incompatibility between transgenic lines in the block, including C5, with these two particular untransformed genotypes, since these two genotypes were the sources of seed used to produce all transgenic lines (Scorza et al., 1994). C5 appears to be self-incompatible, and it is quite possible that sexual incompatibilities within this block of transgenic trees and parental non-transformed lines affect the evaluation of pollen flow. All levels of self-and cross incompatibility can be found in *P. domestica* (Nyéki and Szabó, 1997) thus the self incompatibility of C5 and cross incompatibility with related genotypes is not unanticipated, especially since the female progenitor of C5, 'Bluebyrd', is self-incompatible (Scorza and Fogle, 1999). Incompatibilities within this block may also explain the 1:1 ratio of transgenic to non-transgenic progeny obtained from open-pollination of C5 within this block. Such a ratio indicates that C5 is not self-pollinating

nor being pollinated by other transgenic lines. If self-pollination of transgenics or cross-pollination between transgenic trees was occurring, GUS assays would reveal other than a 1:1 ratio. Evidently, C5 is being pollinated by untransformed trees within the block or from another block at AFRS (see Fig. 26 for locations of other plum blocks at AFRS). An alternative explanation for the apparent lack of cross-pollination between non-transformed and transformed lines within this block is an asynchrony of flowering. Our observations clearly indicate differences in flowering time between genotypes. Without an overlap in bloom cross-pollination will not occur. A third explanation may be related to environmental factors, cold temperatures and rain that could inhibit bee activity during bloom and inhibit pollen flow in any particular year. It should be noted here that within-transgene block transgene pollen flow has been recorded in the PRV-CP transgenic block which is adjacent to the PPV-CP transgenic block. In 1997, 66 fruit were collected from a total of 6 non-transformed trees (B70197 and 'Bluebyrd'). Forty percent of the seedlings obtained were GUS positive.

Since the above analyses of pollen flow within the PPV-CP experimental block were based on GUS assays and not on direct assays of the PPV-CP gene, the linkage between these two transgenes was further confirmed (see section IX *Open-pollination of C5*). Forty-seven seedlings of C5 that were GUS negative were evaluated by DNA blotting for the PPV-CP gene following the methods of Scorza et al. (1994). None of these GUS negative seedlings contained the PPV-CP gene adding additional evidence to the close linkage between these transgenes in C5.

Inter-block pollen flow. Pollen movement was monitored from transgenic blocks of *P. domestica* containing the PRV-CP and PPV-CP at the USDA-ARS Appalachian Fruit Research Station, Kearneysville, WV (APHIS permits 92-191-01 and 95-205-02 and amendments) (**Fig. 27**). To monitor pollen movement from the block (since the two blocks are contiguous they will be referred to as one block) seed were collected from non-transformed plum plantings approximately 520 meters (site 1) and 1076 meters (site 2) (**Fig. 26**) from the test site in 1999, 2000, and 2001. The monitoring evaluated GUS expression in embryos from 350 seeds in each year (1050 seeds total). The 1050 seeds consisted of a total of 850 seeds from 4 trees in site 1 (2 'Bluebyrd' and 2 'Stanley') and 200 seeds from 3 trees in site 2 ('Sentiabrskaja', 'Pozegaca D6', and 'V10') were sampled. These tests revealed that two seeds from 'Bluebyrd' trees in site 1 were positive for GUS and shown by PCR amplification to contain the GUS, NPTII, and PPV-CP genes (**Fig. 28**). Additional tests from these blocks in 2002 (840 seeds, total), 2003 (508 seeds), and 2004 (552 seeds) did not produce positive GUS assay results.

Transgene pollen flow from the PPV-CP transgenic plum block to a non-transformed plum block produced 2 transgenic seeds from a total of 2,950 seeds tested over a period of 6 years. When the pollen flow did occurr, it was at a distance of approximately 520 m. Most likely, bees were the vehicle of pollen movement (Okie and Weinberger, 1996). Besides wild bee populations that may be in the vicinity, bee hives are brought to the field plots of pear and apple at AFRS to improve pollination. Bees and hives are removed following the season of bloom. Apple and pear bloom generally overlap with the bloom period of *P. domestica.*

In order to study transgene flow further six additional "sentinel" sample sites within the boundaries of AFRS has been established (**Fig. 26, Table 8**). Each site

contains nine non-transformed trees. Cultivars 'Seneca' and 'Italian Prune' were selected based on overlapping time of bloom with the transgenic plums, self-incompatibility and cross incompatibility with each other. Since self-incompatible sample trees will not fruit unless pollinated by a compatible pollinator, and since the two sentinel cultivars are incompatible with each other, fruit production will allow us to gauge the amount of pollen inflow in the sample trees and the inflow of transgenic pollen from the transgenic plum blocks. It is not known at this time, however, if these cultivars are compatible with C5 or other transgenic lines and controlled hybridization must be performed when these sentinel cultivars flower to confirm sexual compatibility with transgenic lines. Samples from each sentinel site will consist of all fruit available with a maximum sample size of 500 fruit per 9-tree sample plot. An additional sample site (**Site 3, Fig. 26**) has been established. This block consists of 25 seedling trees that are hybrids of standard *P. domestica* cultivars and Kearneysville *P. domestica* breeding selections. No fruit from sentinel plots will be eaten and seed will not be used for planting. Currently, sites 1, 2, and 3 contain trees that are flowering. Nine-tree sentinel sites containing 'Seneca' and 'Italian Prune' trees will flower in 2006 or 2007.

METHODS TO EVALUATE POLLEN-MEDIATED GENE FLOW

Plum embryos were removed from seed and immersed in a solution consisting of 0.1 M sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.1 % Triton X-100 and 0.8 mM x-glucoronide. Following incubation at 37°C for one hour, embryos were visually assessed for glucoronidase activity as evidenced by blue staining of the tissue. Seed derived from transgenic tree C5 were included in each assay as positive controls. Following immersion in this solution for as long as 3 days, embryos that appeared positive for glucoronidase activity were blotted on tissue paper to remove excess buffer, placed in 1.5 ml microfuge tubes and frozen in liquid nitrogen. Samples were then stored at -80°C until DNA was extracted. DNA from single embryos was extracted following the method of Kobayashi et al. (1998). Each embryo was ground directly in the microfuge tube in which it was frozen using pestles designed to fit standard 1.5 ml tubes. (product # 749521-1590. Nalgene, Rochester, NY). Following spectrophotometric quantitation, 0.1 to 0.5 µg total DNA was subjected to PCR amplification using primers specific for the NPTII, PPV-CP, and GUS genes.

Primer Sequences:

NPTII	forward	5'-cccctcggtatccaattagag-3'
	reverse	5'-cgggggggtgggcgaagaactccag-3'
PPV-CP	forward	5'-aagctgacgaaagagaggacgag-3'
	reverse	5'-ctacactcccctcacaccgaggaa-3'
GUS	forward	5'-gatcagcgttggtgggaaagcgcg-3'
	reverse	5'-caccgaagttcatgccagtccagcg-3'

PCR reactions were performed in a volume of 25 µl and contained buffer with 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$ and 0.001% (w/v) gelatin, (Perkin-Elmer) 200 µM each dNTP's (Promega), 0.75 Units of *Taq* DNA polymerase (Promega), and 500 nM each primer. Thermal cycling consisted of 4 min at 94 °C, followed by 30 cycles of 1 min at 94 °C; 1.5 min at 60 °C (NPTII and PPV-CP) or 65 °C (GUS) for annealing, and 2 min at 72 °C for extension. A final extension of 7 min at 72 °C completed the cycling protocol.

Summary of transgene pollen flow. Pollen flow from transgenic to non-transformed *P. domestica* trees has been documented both within a transgenic block (in the case of PRV-CP transgenics, but not for PPV-CP transgenics) and between a transgenic block and a non-transformed block within the property boundary of the AFRS. This latter case was at a distance of 520 m and occurred at a rate of 0.24% (2 of 850 seeds). No other pollen-mediated gene transfer event has been recorded in 2950 seed tested over a 6-year period. Gene flow between *P. domestica* and other *Prunus* species is unlikely as discussed in section IV *Genetics of P. domestic.* Pollen-mediated gene transfer within *P. domestica* is affected by sexual incompatibilities between genotypes, differences in flowering time, and environmental conditions that affect the activity of insect pollinators. The potential for long-distance spread of transgenic *P. domestica* pollen appears to be low under the conditions of this study and with the genotypes available in the study, considering that genetic incompatibilities appear to exist between some genotypes.

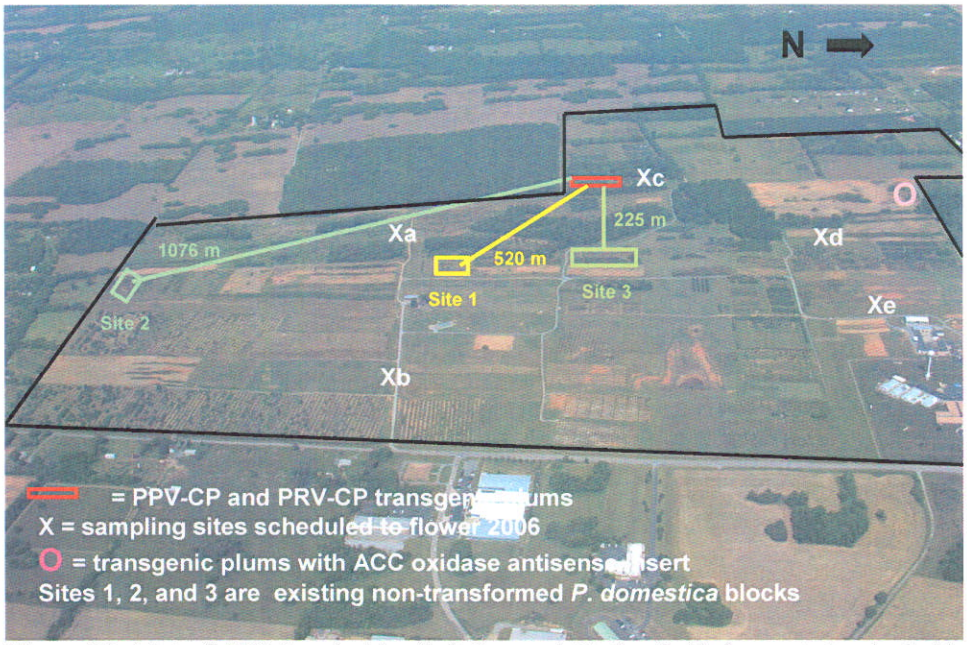

Figure 26. Map of ARS Appalachian fruit Research Station fields (property border in black) indicating viral coat protein transgenic plum (*Prunus domestica* L.) block (red) and non-transgenic plum sample sites 1, 2, and 3. Two transgenic plum seeds were identified in site 1.

Table 7. GPS coordinates of non-transformed plum sentinel sites at AFRS, Kearneysville, WV (see figure 26).

Site	GPS Coordinates	Distance from Transgenic plum block (m)
1	39° 21.055 N; 077° 53.175 W	520
2	39° 20.724 N; 077° 53.336 W	1,076
3	39° 21.178 N; 077° 53.125 W	225
Xa	39° 21.334 N; 077° 53.335 W	600
Xb	39° 20.925 N; 077° 52.961 W	840
Xc	39° 21.334 N; 077° 53.328 W	70
Xd	39° 21.402 N; 077° 53.056 W	720
Xe	39° 21.364 N; 077° 52.837 W	960

A Block 16 - Transgenic plum block (PPV)

Plot map revised 10-15-96
Rows E-W
Trees N-S

Trees planted 10-17-95; underlined planted 5-14-96
= planted fall 1997

39° 21.243N
077° 53.353W

39° 21.255N
077° 53.388W

Tree	Row 1 & 2	3	4	5	6	7	8	9 & 10
17	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP
16	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP
15	Peach Bailey OP	PT19-I	PT20-5	C2-4	C2-27 #	C5-17	PT19-2	Peach Bailey OP
14	Peach Bailey OP	PT4-1	Stanley/ 70146 #	PT23-1	C6-3	C5-14	Bluebyrd-OP	Peach Bailey OP
13	Peach Bailey OP	70146 OP	C3-10 #	C6-6	C2-27	C5-7	PT24-1	Peach Bailey OP
12	Peach Bailey OP	C6-7	C4-3	C4-4	PT4-5	C5-3	C4-12	Peach Bailey OP
11	Peach Bailey OP	PT20-2	Fursts Fruhzwet	Cacanska Rana	PT20-4	C2-15 #	PT20-1	Peach Bailey OP
10	Peach Bailey OP	PT23-2	PT2-5	PT4-2	PT3-3	70146/ Stanley OP	C6-1	Peach Bailey OP
9	Peach Bailey OP	PT5-3	C5-5	C3-13 #	Timpurii Rivers	C2-11 #	PT2-4	Peach Bailey OP
8	Peach Bailey OP	Stanley OP	C2-16 #	PT4-4	PT5-6	C2-21	70146 OP	Peach Bailey OP
7	Peach Bailey OP	C3-16 #	Stanley/ 70146 OP	PT5-5	C4-1	C3-17	PT6-1	Peach Bailey OP
6	Peach Bailey OP	C5-15	PT24-2	C5-13	C5-4	PT23-2	PT3-2	Peach Bailey OP
5	Peach Bailey OP	C2-17 #	PT2-2	70146/ Stanley OP	C5-2	C3-1 #	PT5-4	Peach Bailey OP
4	Peach Bailey OP	PT-6	PT2-6	C3-4	70146/ Stanley OP	C2-14 #	PT4-3	Peach Bailey OP
3	Peach Bailey OP	PT-2	PT3-1	C3-20	Pozegaca D-6 #	Stanley OP	PT6-1	Peach Bailey OP
2	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP
1	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP

39° 21.289N
077° 53.330W

PRV Block

39° 21.335N
077° 53.349W

B **Block 16 - Transgenic plum block (PRV)**

Plot map revised 10-15-96 Trees planted 11-16-92

Rows E-W

Trees N-S

39° 21.289N 077° 53.330W

39° 21.335N 077° 53.349W

Tree	Row 1 & 2	3	4	5	6	7	8	9 & 10
				PPV Block				
13	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP
12	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP
11	Peach Bailey OP							Peach Bailey OP
10	Peach Bailey OP							Peach Bailey OP
9	Peach Bailey OP							Peach Bailey OP
8	Peach Bailey OP							Peach Bailey OP
7	Peach Bailey OP							Peach Bailey OP
6	Peach Bailey OP			St1				Peach Bailey OP
5	Peach Bailey OP				EF1 #2			Peach Bailey OP
4	Peach Bailey OP			EF1 #59	B69158/ Stanley OP			Peach Bailey OP
3	Peach Bailey OP							Peach Bailey OP
2	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP
1	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP	Peach Bailey OP

39° 21.323N 077° 53.316W

39° 21.302N 077° 53.365W

Figure 27. Transgenic *P. domestica* plum blocks at AFRS, Kearneysville, WV APHIS. **A.** Trees containing *Plum pox virus* coat protein (PPV-CP) gene planted in 1995, in 1996 (underlined trees), and in 1997 (# marked trees) under APHIS permit 95-205-02. Trees highlighted in red are transgenic. Non-highlighted trees are non-transgenic controls. The planting is surrounded by a double row of seedling peach trees which are sexually incompatible with *P. domestica* plum. GPS coordinates are indicated. **B.** Trees containing *Papaya ringspot virus* coat protein (PRV-CP) gene planted in 1992 under APHIS permit 92-191-01. Trees highlighted in red are transgenic. Non-highlighted trees are non-transgenic controls. The planting is surrounded by a double row of seedling peach trees which are sexually incompatible with *P. domestica* plum. GPS coordinates are indicated. Tree spacings in both plantings are 6 meters between rows, 6 meters between trees.

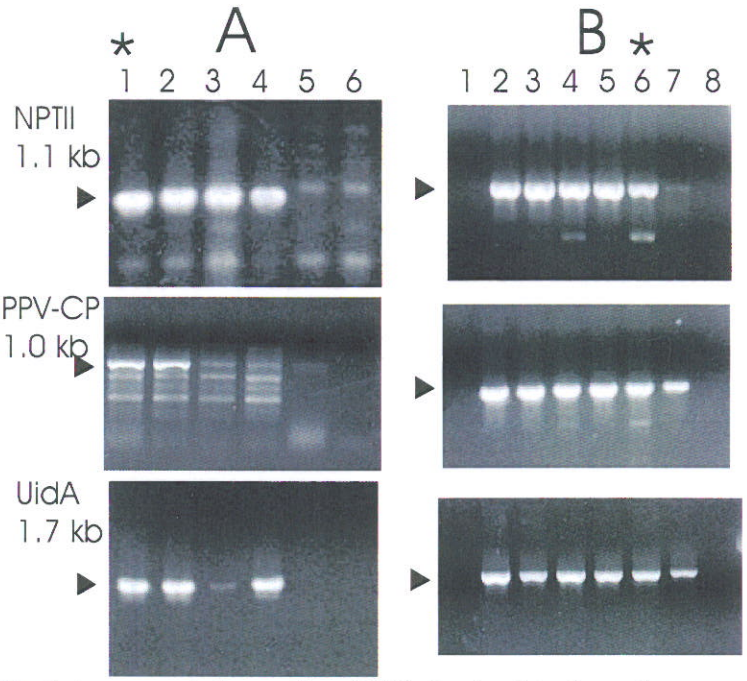

Figure 28. Polymerase chain reaction (PCR) for the detection of transgenes (NPTII, PPV-CP, and GUS) in seed collected from open-pollinated non-transformed *P. domestica* plum from 2 seeds collected in site 1 (Fig. 26, Table 7) of a total of 850 seeds collected from site 1.over three years; 1999, 2000, 2001. (A) *Lane 1, GUS positive 'Bluebyrd' open-pollinated #1 (site 1). Lanes 2-4, GUS-positive C5 open-pollinated. Lanes 5 and 6, GUS-negative 'Bluebyrd' open-pollinated. (B) Lane 1, GUS Negative 'Bluebyrd' open-pollinated. Lanes 2-5, GUS-positive C5 open-pollinated. *Lane 6, GUS-positive 'Bluebyrd' open-pollinated #2 (site 1). Lane 7, C5 parent leaf sample. Lane 8, no template control.

XIII. Environmental Consequences of Introduction of Transformed Cultivar.

In order to evaluate the potential for major alterations in the insect fauna associated with trees expressing *plum pox* virus coat protein or marker transgenes in 2001 fruit were harvested in the PPV-CP transgenic block at AFRS, Kearneysville, WV (**Fig. 27**). Sampled trees were left unsprayed. Trees sampled were three to five trees of each PPV-CP transgenic line C3 (moderate PPV-CP expresser), C4 (high PPV-CP expresser), C5 and C6 (no expression of PPV-CP) and one tree each of three clones that expressed marker gene proteins (*nptII* and GUS) but contained no PPV-CP transgene, and non-transformed 'Stanley' (2 trees), selection 70146 (two trees), 'Stanley' open-pollinated seedling (1 tree), and 70147 open-pollinated seedling (one tree). Fifty fruit were sampled at random from each test tree at maturity and individually evaluated for damage by plum curculio (*Conotrachelus nenuphar*). Damage was rated as feeding, larval, and/or ovipositional damage and calculated as a percent of total fruit exhibiting damage. Stinkbug (*Acrosternum hilare*, *Euschistus servus*, *E. tristigmus*), and Lygus bug (*Lygus herperus*) damage and external damage by unidentified chewing insects ("worm damage") was also recorded. Data was subjected to analysis of variance. Results are

presented in **Table 8.** There appears to be no consistent association of fruit damage with either transgenic trees expressing high levels of PPV-CP, moderate levels of PPV-CP, or marker gene expression. Variability in damage was high. While this study presents only a "snapshot" in time, there does not appear to be an effect of the transgenes, PPV-CP or marker genes, that is greater than the natural variability that may be found in an unsprayed plum planting.

Table 8. Fruit harvest insect damage assessment in a mixed PPV-CP transgenic plum and non-transformed plum (*P. domestica*) block, 2001.

	% PC Feeding Damage			% PC Larval Damage			% PC Ovipositional Damage			% Stinkbug/Lygus Damage			% External Feeding Damage		
Genotypes	Mean	SE		Mean	SE		Mean	SE		Mean	SE		Mean	SE	
NT	3.00	0.86	AB	6.33	2.85	A	2.67	0.99	AB	0.00	0.00	A	0.67	0.42	B
C3	5.00	2.38	AB	0.00	0.00	B	0.00	0.00	B	0.50	0.50	A	2.00	1.41	B
C4	1.00	1.00	AB	0.00	0.00	B	8.00	8.00	A	1.00	1.00	A	1.00	1.00	B
C5	1.20	1.20	AB	0.00	0.00	B	0.40	0.40	B	0.80	0.49	A	6.21	1.56	A
C6	8.00	5.29	A	3.33	1.33	AB	3.33	0.67	AB	0.00	0.00	A	0.00	0.00	B
MO	0.00	0.00	B	0.50	0.50	B	2.00	1.41	B	0.00	0.00	A	0.50	0.50	B

PC = Plum Curculio NT = Non-transformed MO = Marker Genes Only

XIV. Intended Cultivation Area, Cultivation Practices, Likely Deployment

Currently (2005), PPV in the U.S. is under an eradication program. Few newly infected trees have been discovered in the Pennsylvania quarantine area and no other infected trees have been discovered in the U.S. Under these conditions it is not expected that there will be a significant area of cultivation of C5 plum in the U.S. The situation would change if PPV were to be widely introduced into the U.S., particularly in California where there is a considerable acreage of *P. domestica*. At this time deployment in the U.S. is expected to be mainly in breeding programs where C5 can be used as a parent for developing high fruit quality PPV-resistant germplasm and cultivars for deployment in the event that PPV should re-enter the U.S. and become a serious threat to U.S. plum production.

Cultivation practices for C5 are not expected to differ between C5 and non-transgenic *P. domestica* plums. An example of cultivation practice recommendations for plum can be found at http://eesc.orst.edu/agcomwebfile/edmat/EC773.pdf (Stebbins, 1993).

XV. Adverse or Unintended Consequences of Introduction of the New Cultivar

There are no known or anticipated adverse consequences of the introduction of the new cultivar. *P. domestica* is not considered a weedy species, in the absence or presence of PPV therefore resistance to PPV would not be expected to make it so. The transfer of the PPV-CP transgene to other species is unlikely (see section IV. Genetics of *P. domestica*) and if transfer were to take place there is no reason to believe that resistance to PPV would cause weediness. In terms of increased fitness of C5, the only potential for the expression of increased fitness could occur in the event of an epidemic of a severe strain of PPV where C5 could produce more fruit to maturity than plum trees susceptible to PPV.

Unintended effects of the PPV-CP transgene are not likely for at least 2 major reasons. First, the transgene produces no PPV coat protein. Without protein there is no apparent mechanism by which non-target fauna or flora could be affected. Secondly, non-transformed plums have been shown to naturally produce siRNA (the short 22 nucleotide species) upon infection with PPV (Hily et al., 2005). Therefore siRNA is a natural system for virus defense in plum and not outside of the realm of what can be found already existing in the natural environment.

The marker genes NPTII and GUS are expressed in C5 plant tissues. These proteins have been classified as exempt from the requirement of a tolerance in or on all raw agricultural commodities when used as plant-pesticide inert ingredients (59 FR 49353, Sept. 28, 1994 and 66 Fed. Reg. 42957-42962, August 16, 2001 for NPTII and GUS, respectively). While C5 contains several copies of the DNA region originating from plasmid pBR322 that contain the β-lactamase gene, the gene is interrupted by a fragment containing the cos site and is non-functional. The potential for mutation in which the *cos* site could be eliminated to produce a functional β-lactamase gene was tested in *E. coli* containing the plasmid originally used to engineer C5. These tests indicated a rate of mutation to a functional β-lactamase gene of 5 events in 1,000,000,000 *E. coli* cells (see section VIII. Characterization of the DNA inserted into C5). It should be emphasized that these results were taken from work with *E. coli* and not from C5 itself where cell multiplication and hence the potential mutation rate is many orders of magnitude below that of *E. coli.* As such, the excision of the *cos* site from the β-lactamase gene in C5 would be expected to be an exceedingly rare event.

The presence of the transgene is not expected to alter the breeding behavior of the plum tree in comparison to the breeding behavior of any sibling of C5. Studies using C5 as a parent in breeding reported in this application (Section XI) indicate normal breeding behavior.

XVI. Characterization of the virus resistant C5 plum.

Equivalency. C5 plum is typical for cultivated *P. domestica* plums (**Table 9**). Growth habit can be classified as upright. Spur formation is moderate, vigor is moderate and root sucker production is moderate when planted on its own roots. C5 is self-sterile which is not unusual since naturally in *P. domestica* every degree of fertility, from entirely self-sterile to highly self-fertile can be found (Nyeki and Szabo, 1997). Productivity is good in the presence of compatible pollinators. C5 displays no characteristics that would not be expected from any typical *P. domestica* tree. Fruit quality in terms of flavor, size and color are excellent. Measurements of fruit quality traits are influenced by fruit maturity, which is not a fixed point but rather a continuously advancing fruit developmental characteristic. Environmental conditions such as temperature, rainfall, and solar radiation influence fruit quality as do growing conditions such as fertility, pruning, and rootstock. Therefore, a wide range in fruit quality values can be obtained both between and within varieties. With this stated, measurements of morphological and horticultural characteristics of C5 indicate that it is well within the range of typical *P. domestica* plum varieties currently available in the market (**Table 9**). Fruit compositional analyses also indicate that C5 is typical for *P. domestica* plums (**Table 10**).

Table 9. Tree, foliage, flower, fruit, and stone characteristics of C5 and other *P. domestica* plum cultivars based on plant patent descriptions except for 'Bluebyrd', the non-patented female progenitor of C5. (The male progenitor of C5 is unknown.)

[1] Royal Horticultural Society color chart designations. [2] Dictionary of Color (Maerz and Paul, 1950). [3] Color Name Chart of The Inter-Society Color Council, National Bureau of Standards. [4] Italicized numbers are color designations with waxy bloom on fruit. Non-italicized are with waxy bloom rubbed off.

	C5	**Bluebyrd**	**Red Sun**	**D6N-72**	**Tulare Giant**	**Sutter**	**Rosy Jewel**	**Jayfree**	**Emperor**
Tree									
Canopy height (m)	4.9	3.9	"typical"	5.5	4.5	5.6	3.5	3.5	"Large upright"
Canopy width (m)	2.7	1.4	"	4.3	4	4.3	4.4	5	
Fertility	Self-unfruitful	Self-unfruitful	Self-unfruitful	Self-fruitful	No info.	Partially self-fruitful	No info.	No info.	No info.
Productivity	14.4 kg/tree@8 yrs	v. productive	v. good	v. productive	high	good	350 24 lb. boxes/a	heavy	v. productive
Leaves									
Length (mm)	78-114	66-85	25-70	104-141	109-154	111-134	"medium"	80-85	113
Width (mm)	49-68	43-57	15-20	47-63	55-68	52-64	"	30-60	63
Flowers									
Color	white	white	white	white	white	white	white	white	white
Petal length (mm)	10	No info.	8	10-12	11-14	10-13	5-10	7	10
Petal width (mm)	7	"	6	8-10	10-12	7-9	15	5	8
Pollen	present	present	No info.	abundant	abundant	abundant	No info.	present	abundant
Pollen color	11A[1]	No info.	"	13A[1]	14A[1]	14B[1]	"	No info.	10-L-3 [2]
Fruit									
Diameter (mm)	43	40	40	37-45	40-53	29-34	55-58	43-48	55
Length (mm)	52	50	43	45-53	54-68	47-51	48-63	55-58	71
Skin color	*91B, 97B[1,4];* 103A, N186A	***92B[1,4];*** N92	Dark red. 17 v.d.r.[3]	*76A, 85A, 91A[4];* 180A, 187A[1]	*97B[4]*;187A[1]	*98D,185C[4];* 184B, 79A[1]	P1. 48-H-12[2]	187A, 79A	48-H-12[2]
Flesh color	6A, 6C, 151A[1]	16C, 13B, 13C	Light yellow. 72 d.oy[3].	15B-26A[1]	20B,20A[1]	20A, 22A[1]	P1. 19-C-2, P1. 19-C1[2]	16A	12-K-7[2]
Brix (soluble solids)	22	20	No info.	19	20	24-25	14	No info.	Less than 22
Stone									
Attachment to flesh	free	free	free	free	Semi-free	Semi-free	No info.	Semi-free	cling
Length (mm)	27	24	8 (sic)	28-32	28-33	25-29	25-30	24	37-40
width	17	**13**	16	12-15	14-17	10-12	18	22	19-21

Table 10. Plum fruit compositional analysis.

	Bluebyrd	**C5**	Early Italian	Green Gage	Reine Claude	Stanley	Units	Method Reference
Ash	4.01	**2.91**	3.77	3.04	3.23	3.94	%	AOAC 923.03
Fat	0.27	**0.21**	0.17	0.21	0.31	0.26	%	Acid hydrolysisAOAC 16th Ed.
Moisture	79.02	**85.02**	82.07	81.4	82.48	79.7	%	Vacuum oven 100 deg. 5 hr. AOAC927.05
Acidity	1.3	**1.2**	0.6	0.9	1.2	0.4	%	as Acetic Acid
Total Dietary Fiber	2.58	**2.74**	2.29	2.58	2.5	2.18	%	AOAC 985.25 16th Ed.
Carbohydrates	16.06	**10.83**	12.94	14.71	13.26	15.56	%	Calculated
Vitamin A	<17.8	**<13.2**	<15.5	<15.9	<15.1	<17.1	IU/100g	AOAC 974.29 HPLC (Mod)
Vitamin C	6.2	**8**	4.3	<0.4	2.7	9.5	mg/100g	AOAC 967.21, 984.26 MVTL
Total Sugar	10.43	**8.25**	9.3	10.72	8.84	10.48	g/100g	
Glucose	2.29	**3.82**	3.77	4.35	3.43	3.52	g/100g	
Sucrose	6.58	**2.6**	4.52	4.37	4.39	4.91	g/100g	
Lactose	<0.1	**<0.1**	<0.1	<0.1	<0.1	<0.01	g/100g	AOAC 15th Ed. MVTL
Maltose	<0.1	**<0.1**	<0.1	<0.1	<0.1	<0.1	g/100g	
Fructose	1.56	**1.83**	1.01	2	1.02	2.05	g/100g	
Calcium	<0.01	**<0.01**	<0.01	<0.01	<0.01	<0.01	%	AOAC 985.01
Magnesium	<0.01	**<0.01**	<0.01	<0.01	<0.01	<0.01	%	AOAC 985.01
Sodium	<0.01	**<0.01**	<0.01	<0.01	<0.01	<0.01	%	AOAC 985.01
Potassium	0.17	**0.21**	0.19	0.18	0.19	0.15	%	AOAC 985.01
Iron	1.521	**1.508**	1.177	0.737	1.766	1.622	mg/Kg	EPA 6010
Protein	0.64	**1.03**	1.05	0.64	0.72	0.54	%	N x 6.25 AOAC 988.05
Starch	0.04	**<0.01**	0.04	0.23	0.08	<0.01	%	
Thiamine	<0.44	**0.43**	0.47	<0.4	<0.38	<0.43	mg/Kg	AOAC 942.23 17th Ed.
Riboflavin	<1.11	**<0.83**	<0.97	<0.99	<0.95	1.07	mg/Kg	AOAC 970.65
Niacin	11.8	**20.8**	<3.88	17.9	24.1	11.7	mg/Kg	AOAC 960.46/944.13 (Mod)
Antioxidant capacity	20	**13**	15	17	16	19	umole TE/g	ORAC water soluble antioxidant capacity[1]
Phenolics	1.59	**1.18**	1.46	1.6	1.31	1.63	mg/g	gallic acid equivalent

All analyses by Minnesota Valley Testing Laboratories, Inc. New Ulm, MN except antioxidant capacity and phenolics which were by Brunswick Laboratories Wareham, MA.

[1] The acceptable precision of the ORAC assay is 15% relative standard deviation (Ou et al, 2001).

The following is a detailed description of the botanical and pomological characteristics of C5 plum. Color data are presented in Royal Horticultural Society (RHS) Color Chart designations. Where dimensions, sizes, color, and other characteristics are given, such characteristics are approximations of averages set forth as accurately as practicable. The descriptions reported are from specimens grown at Kearneysville, West Virginia and are part of the 'HoneySweet' plum patent application.

Tree:
Size – (at 7 years) height 4.9 m; canopy diameter 2.7 m
Vigor - current season shoot length 8 yr trees 41.3 cm.
Growth - upright, branch angles from main limbs 54°
Productivity - 14.4 kg/tree 8 yr trees.
Bearing - Regular, no tendency towards alternate bearing.

Trunk:
Size - (at 7 years, 15-20 cm above soil line) diameter 16.5 cm; circumference 51.3 cm.
Color - RHS 197A to RHS 197D.

Branches:
Size - average diameter of 1-year branches 3.9 mm; 2-year branches 4.6 mm.
Texture - Smooth to medium rough.
Spur development - 0.44 spurs/cm on 2-year branches, spur length 5-7 mm.
Color - RHS 197A-D.
Lenticels - (5-6 yr. branches) 5mm long, 2/cm^2, color RHS 167C.
Shoots from roots (root suckers) - On 8-year-old self-rooted trees an average of 88 root suckers.

Leaves:
Size - Average length 92 mm, average width 45 mm.
Texture.—Glabrous to somewhat rough.
Glands - Two, small, round average width 0.44 mm, length 0.52 mm; color RHS 137B .
Margin - Dentate.
Form - Obovate - pointed.
Color - Adaxial (upper) surface—RHS 139A . Abaxial (lower) surface—RHS 137B to 137A.
Petiole - length 11.8 mm, width 1.4 mm, thickness 1.3mm; color RHS 137B.

Flowers:
Flowers are complete, perfect, perigynous. Five sepals form a hypanthium cup averaging 3.1 mm in height and 3.7 mm in diameter. Five white petals are attached to hypanthium. Stamens, average 15 per flower, are attached to interior of hypanthium at or below the rim of the hypanthium cup. Pistil is superior averaging 10.9 mm in length. Stigma is round averaging 0.86 mm in diameter. Style averages 7.5 mm in length and 0.4 mm in diameter. Ovary averages 2.5 mm in height and 1.6 mm in diameter. No scent is detectable.

Bloom period - Variable depending on weather, late March to mid-April in the Eastern Panhandle of West Virginia.
Petal color - white
Petal size - 6.9 X 9.6 mm
Filaments - 7.7 mm long; color RHS 155D.
Pilsil - 10.9 mm
Pedicel - 8.8 mm
Anther color - RHS 13A
Style color - RHS 137B
Sepal color - RHS 143C
Pollen - Present; color - RHS 11A
Fertility - self-sterile

Fruit:
Maturity when described - Shipping Ripe-Eating Ripe.
Average date of Harvest - Mid August to Early September, in Kearneysville, West Virginia.
Size - Medium to Large. Average size is 43 mm diameter right angle to suture x 45 mm diameter across suture x 52 mm long; average weight is 60 grams.
Yield - In the experimental plot limited yield data has been collected. In one sampling year an average yield over nine sampled trees was 13.5 kg/tree.

Flesh:
Ripens - Evenly.
Texture - Firm.
Fibers - Small, few, tender.
Juice - Moderate at eating-ripe .
Aroma - Moderate.
Eating quality - Sweet, excellent. Brix of ripe fruit averages 21.5° depending on maturity at harvest.
Color - RHS 6A to RHS 6C to RHS 151A depending on stage of ripeness.
Pit cavity color same as flesh color.

Skin:
Tendency to crack - None.
Color - With wax bloom RHS 91B to RHS 97B, with wax bloom removed RHS 103A to RHS N186A
Use - Dessert
Market - Local and long distance.

Stone:
Type - Freestone – slight cling.
Size - Medium. Average length 27 millimeters, average width 17 mm 29.8mm, average thickness 10 mm.

Form - Ovate/asymmetrical.
Tendency to split - None
Color - RHS 165C.

Summary of C5 tree and fruit characteristics. C5 trees are of an upright growth habit, have good vigor and yield well in the presence of compatible pollinators. Fruit are sweet, highly flavored, and of medium to large size. The potential for drying has not been evaluated but sugar content would indicate the potential for drying. In all tree and fruit characters observed C5 appears to possess those qualities necessary for a commercial *P. domestica* plum cultivar.

Figure 29. Fruit and leaves of C5 at maturity.

Figure 30. Own-rooted tree of C5 at 8 years AFRS, Kearneysville, WV (height approximately 5m).

XVII. LITERATURE CITED

Abernathy, D., Zhebentyayeva, T., Vilanova, S., Badenes, M.L., Salava, J., Polák, J., Krska, B., Damsteegt, V.D. 2004. Molecular genetic mapping of the Plum pox virus resistance genes in apricot. Acta Hort. 657: 283-288.

An, G. Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells. Plant Physiol. 81:86-91.

Al Rwahnih, M., Myrta, A., Di Terlizzi, B. and Boscia, D. 2001. First record of plum pox virus in Jordan. Acta Hort 550:141-144.

Asensio, M., Saenz, P., García, J.A. and Cambra, M. 1999. Plum pox virus isolate 3.3 RB/GF-Mp15GF coat protein mRNA, partial cds. GenBank AF172348 (GI: 6049497).

Atanassov, D. 1932. Plum pox. A new virus disease. Ann Univ Sofia, Fac Agric Silvic 11: 49-69.

Avinent L., Hermoso de Mendoza, A. and Lácer, G. 1993. Comparison of sampling methods to evaluate aphid populations (Homoptera, Aphidinea) alighting on apricot trees. Agronomie 13:609-613.

Bailey, J.M. and Davidson, N. 1975. Methyl mercury as a reversible denaturing agent for agarose gel electrophoresis. Anal Biochem 70:75-85.

Barker, R.F., Idler, K.C., Thompson, D.V. and Kemp, J.D. 1983. Nucleotide sequence of the T-DNA region from the Agrobacterium tumefaciens octapine Ti plasmid pTi15955. Plant Mol. Biol. 2:335-350.

Baulcombe, D.C. 1996. Mechanisms of pathogen-derived resistance to viruses in transgenic plants. Plant Cell 8:1833-1844.

Beachy, R.N., Loesch-Fries, S. and Tumer, N.E. 1990. Coat protein-mediated resistance against virus infection. Ann. Rev. Phytopath. 28:451-474.

Beck, E., Ludwig, G., Auerswald, E.A., Reiss, B. and Schaller, H. 1982. Nucleotide sequence and exact localisation of the neomycin phosphotransferase gene from the transposon Tn5. Gene 19:327-336.

Bennett, M.D. and Leitch, I.J. 1995. Nuclear DNA amounts in angiosperms. Ann. Bot. 76:113-176.

Berger, P.H., Hunt, A.G., Domier, L.L., Hellman, G.M., Strm, Y., Thronbury, D.W. and Pirone, T.P. 1989. Expression in transgenic plants of a viral gene product that

mediates insect transmission of potyvirus. Proc. Natl Acad Sci USA 86:8402-8406.

Bernstein E., Caudy, A.A., Hammond, S.M. and Hannon, G.J. 2001. Role for a bidentate ribonuclease in the initiation step of RNA. Nature 409:363-366.

Bevan, M., Barnes, W.M. and Chilton, M.D. 1983. Structure and transcription of the nopaline synthase gene region of T-DNA. Nucleic Acid Res. 11:369-385.

Botu, M., Sarpe, C., Cosmulescu, S. and Botu, I. 2002. The genetic control of pollen fertility, pollenizing and fruit set for the *Prunus domestica* L. plum cultivars. Acta Hort. 577:139-145.

Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 71:248-254.

Callahan, A.M., Morgens, P.H., Wright, P. and Nichols, Jr. K.E. 1992. Comparisons of pch313 (pTOM13 homolog) RNA accumulation during fruit softening and wounding of two phenotypically different peach cultivars. Plant Physiol. 100:482-488.

Cambra, M., Asensio, M., Gorris, M.T., Pérez, E., Camarasa, E., García, J.A., López-Moya, J.J., López Abella, D., Vela, C. and Sanz, A. 1994. Detection of plum pox potyvirus using monoclonal antibodies to structural and non structural proteins. Bul. OEPP/EPPO Bul. 24:569–577.

Cambra M., Gorris, M.T., Marroquín, C., Román, M.P., Olmos, A., Martínez, M.C., Hermoso de Mendoza, A., López, A. and Navarro, L. 2000. Incidence and epidemiology of *Citrus tristeza virus* in the Valencia Community of Spain. Virus Res. 71:85-95.

Coutanceau, M. 1953. Arboricuture fruitiere: technique et economie des cultures de rosacees fruitieres ligneuses. Bailliere. Paris.

Crane, M.B. and Lawrence, W.J. 152. The genetics of garden plants. 3rd ed. Macmillan. London.

Cullinan, F.P. 1937. Improvement of stone fruits. Yearbook Agric. USDA pp. 665-748.

Damsteegt, V.D., Scorza, R., Gildow, F.E., Schneider, W.L., Stone, A.L. and Luster, D.G. 2004. Comparative host range of U.S. isolates of Plum pox virus among Prunus and other woody plant species following graft inoculation or aphid transmission. Phytopathology 94:S24.

Darlington, C.D. and Ammal Janaki, E.K. 1945. Chromosome Atlas of Flowering Plants. London. Allen Unwin LTD p. 149.

Davies, G.J., Sheikh, M.A., Ratcliff, O.J., Coupland, G. and Furner, I.J. 1997. Genetics of homology-dependent gene silencing in Arabidopsis; a role for methylation. Plant J. 12:791-804.

Depicker A, Stachel S, Dhaese P, Zambryski P, Goodman HM. 1982. Nopaline synthase: transcript mapping and DNA sequence. Journal of Molecular and Applied Genetics 1: 561–573.

Dondini, L., Costa, F., Tataranni, G., Tartarini, S., and Sansavini, S. 2004. Cloning of apricot RGAs (Resistance Gene Analogs) and development of molecular markers associated with Sharka (PPV) resistance. J. Hort. Sci. and Biotech. 79: 729-734.

Doyle, J.J. and Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Dunez, J. and Sutic, D. Plum pox virus. In: Smith, I.M., Dunez, J., Elliot, R.A., Phillips, D.H. and Arches, S.A. (Eds) 1988. European Handbook of Plant Diseases. Blackwell, London, 44-46.

Elbashir, S.M., Lendeckel, W. and Tuschl, T. 2001. RNA interference is mediated by 21- and 22- nucleotide RNAs. Gene Dev. 15:188-200.

English, J.J., Mueller, E. and Baulcombe, D.C. 1996. Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. Plant Cell. 8:179-188.

FAOSTAT data, 2004. http://faostat.fao.org/faostat/notes/citation.htm "last updated February 2004".

Fitch, M.M.M., Manshardt, R.M., Gonsalves, D., Slightom, J.L. and Sanford, J.C. 1990. Stable transformation of papaya via microprojectile bombardment. Plant Cell Repts. 9:189-194.

Fogle, H.W. 1975. Cherries. In. Janick, J. and Moore, J.N. (eds) Advances in Fruit Breeding. Purdue University Press, West Lafayette IN.

Georgi, L.L., Wang, Y., Yvergniaux, D., Ormsbee, T., Iñigo, M., Reighard, G., Abbott, A.G. 2002. Construction of a BAC library and its application to the identification of simple sequence repeats in peach [*Prunus persica* (L.) Batsch]. Theor Appl Genet (2002) 105:1151–1158

Gianessi, L. 2004. Biotechology Expands Pest-Management Options for Horticulture. Calif. Ag. 58:94-95.

Gianessi, L.P., Silvers, C.S., Sankula, S. and Carpenter, J.E. 2002. Viral Resistant Stone Fruit. In: Plant Biotechnology: Current and Potential Impact for Improving Pest Management in U.S. Agriculture. National Center for Food and Agricultural Policy. Washington, D.C.

Gleason, H. and Cronquist, A. 1991. Manual of Vascular Plants of Northeasern United States and Adjacent Canada. 2nd Ed. New York Botanical Garden, NY. 910 p.

Great Plains Flora Association. 1986. Flora of the Great Plains. University Press of Kansas, Lawrence, KS. 1392 p.

Guo, H.S., Lopez-Moya, J.J. and Garcia, J.A. 1999. Mitotic stability of infection-induced resistance to plum pox potyvirus associated with transgene silencing and DNA methylation. MPMI 12:103-111.

Hamilton, A.J. and Baulcombe, D.C. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:950-952.

Hammond, S.M., Bernstein, E., Beach, D. and Hannon, G.J. 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404:293-296.

Hily, J.M., Scorza, R., Malinowski, T., Zawadzka, B. and 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, J.M., Scorza, R., Webb, K., and Ravelonandro, M. 2005. Accumulation of the long class of siRNA is associated with resistance to *Plum pox virus* in a transgenic woody perennial plum tree. MPMI 18: 794-799.

Ingelbrecht, I.L., Van Houdt, H., Van Montagu, M. and Depicker, A. 1994. Post-transcriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc Natl Acad Sci USA 91:10502-10506.

Jan F.J., Pang S.Z., Fagoaga C., Gonsalves D. 1999. Turnip mosaic potyvirus resistance in *Nicotiana benthamiana* derived by post-transcriptional gene silencing. Transgenic Res. 8: 203-213.

Janick, J. and Moore, J.N. 1975. Advances in Fruit Breeding. Purdue University Press, West Lafayette, IN.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol Biol Reptr 5:387-405.

Jones, L., Hamilton, A.J, Voinnet, O., Thomas, C.L., Maule, A.J. and Baulcombe, D.C. 1999. RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. Plant Cell 12:2291-2301.

Kegler, H., Fuchs, E., Gruntzig, M. and Shwarz, S. 1998. Some results of 50 years of research on the resistance to Plum pox virus. Acta Virol 42:200-215.

Kerlan, C. and Dunez, J. Diférenciation biologique et sérologique de souches du virus de la Sharka. Ann.Phytopath 11: 241-250.

Kobayashi, N., Horikoshi, T., Katsuyama, H., Handa, T. and Takayanagi, K. 1998. A simple and efficient DNA extraction method for plants, especially woody plants. Plant Tissue Culture and Biotech 4:76-80.

Kohli, A., Gahakawa, D., Vain, P., Laurie, D.A. and Christou, P. 1999. Transgene expression in rice engineered through particle bombardment: molecular factors controlling stable expression and transgene silencing. Planta 208:88-97.

Korschineck, I., Himmler, G., Sagl, R., Steinkellner, H. and Katinger, H.W.D. 1991. A PCR membrane spot assay for the detection of plum pox virus RNA in bark of infected trees. J. Virol. Methods 31:139-146.

Kunze, L. and Krczal, H. 1971. Transmission of sharka virus by aphids. Ann. Phytopathol HS 355-260.

Layne, R.E.C. 1983. Hybridization. pp. 48-65. In: Methods in Fruit Breeding (Moore, J.N. and Janick, J. eds.) Purdue Univ. Press, West Lafayette, IN.

Layne, R.E.C. and Sherman, W.B. 1986. Interspecific hybridization of *Prunus*. HortScience 21:48-51.

Levy, L., Damsteegt, V. and Welliver, R. 2000. First Report of *Plum Pox Virus* (Sharka Disease) in *Prunus persica* in the United States. Plant Dis 84:202.

Lindbo J.A., Dougherty W.G. 1992. Pathogen-derived resistance to a potyvirus: immune and resistant phenotype in transgenic tobacco expressing altered form of potyvirus coat protein nucleotid sequence. MPMI 5: 144-153.

Ling, K.S., Namba, S., Gonsalves, C., Slightom, J.L. and Gonsalves, D. 1991. Protection against detrimental effects of Potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. Bio/Technology 9:752-758.

López-Moya, J.J., Sanz, A., Cambra, M., Gorris, M.T., Anaya, G., Miguet, J.G., Cortés, E. and López-Abellá, D. 1994. Production and characterization of monoclonal antibodies to plum pox virus and their use in differentiation of Mediterranean isolates. Arch. Virol. 135: 293-304.

Malinowski, T., Zawadzka ,B., Ravelonandro, M. and Scorza, R. 1998. Preliminary report on the apparent breaking of resistance of a transgenic plum by chip bud inoculation of plum pox virus PPV-S. Acta virologica, 42:241-243.

Malinowski T., Cambra M., Capote N., Zawadzka B., Gorris M.T., Scorza R. and Ravelonandro M. 2006. Field trials of plum clones (*Prunus domestica* L) transformed with the *Plum pox virus* coat protein (PPV-CP) gene. Plant Dis. (In press)

Mante, S., Morgens, P.H., Scorza, R., Cordts, J.M. and Callahan, A.M. 1991. *Agrobacterium*-mediated transformation of plum (*Prunus domestica* L.) hypocotyls slices and regeneration of transgenic plants. BioTechnology 9:853-857.

Mehlenbacher, S.A., Cociu, V. and Hough, L.F. 1991. Apricots. In: Moore, J.N. and Ballington, R.J. (eds) Genetic resources of temperate fruit and nut crops. ISHS Wageningen pp. 65-107.

Missouri Botanical Garden. 1990. Index to plant chromosome numbers 1986-1987. Monographs in Systematic Botany. Vol 30:169.

Missouri Botanical Garden. 1991. Index to plant chromosome numbers 1988-1989. Monographs in Systematic Botany. Vol 30:172.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Navratil, M., Sararova, D., Karesova, R. and Petrizk, K. 2005. First incidence of Plum pox virus on apricot trees in China. Plant Dis. 89:338.

Nemchinov, L. and Hadidi, A. 1996. Characterization of the sour cherry strain of plum pox virus. Phytopathology 86:575-580.

Nemchinov, L., Hadidi, A., Maiss, H., Cambra, M., Candresse, T. and Damsteegt, V. 1996. Sour cherry strain of plum pox potyvirus (PPV): molecular and serological evidence of a new subgroup of PPV strains. Phytopathology 86:1215-1221.

Neumüller M., Hartmann W., Stösser R., 2005. The hypersensitivity of European Plum against *Plum pox virus* (PPV) as a promising mechanism of resistance. Phytopathologia Polonica 36: 77-84.

Nyéki , J. and Szabó, Z. 1997. Cross-incompatibility in stone fruits. Acta Hort. 437:213-217.

Odell, J.T., Nagy, C. and Chua, N.H. 1985. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810-812.

Okie, W.R. and Weinberger, J.H. 1996. Plums. pp:559-607. In: Fruit Breeding, Volume I: Tree and Tropical Fruits, edited by J. Janick and J.N. Moore. John Wiley and Sons, Inc. New York.

Olmos, A., Cambra, M., Dasi, M.A., Candresse, T., Esteban, O., Gorris, M.T. and Asensio, M. 1997. Simultaneous detection and typing of plum pox potyvirus (PPV) isolates by heminested-PCR and PCR-ELISA. J. Virol. Methods, 68:127-137.

Organization for Economic Co-operation and Development (OECD). 2002. Consensus document on the biology of Prunus Sp. (Stone Fruits). Series on Harmonization of Regulatory Oversight in Biology, No. 24. OECD, Paris. 40 p. (http://www.oecd.org/ehs/)

Ou, B., Hampsch-Woodill, M., Prior, R.L.. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J. Ag and Food Chem. 49: 4619-4626.

Powell-Abel, P., Nelson, R.S., De, B., Hoffman, N., Rogers, S.G., Fraley, R.T. and Beachy, R.N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743.

Ravelonandro, M., Briard, P. and Scorza, R. 2001a. Significant resistance of transgenic plums against the four serotypes of plum pox potyvirus. Acta Hort 550:431-435.

Ravelonandro, M., Briard, P., Renaud, R. and Scorza, R. 2001b. Transgene-based resistance to plum pox virus (Sharka disease) is transferred through interspecific hybridization in *Prunus*. Acta Hort. 546:569-574.

Ravelonandro, M., Dunez, J., Scorza, R. and Labonne, G. 1998. Challenging transgenic plums expressing potyvirus coat protein genes with viruliferous aphids. Acta Hort. 472:413-420.

Ravelonandro, M., Monsion, M., Delbos, R. and Dunez, J. 1993. Variable resistance to plum pox virus and potato virus Y infection in transgenic *Nicotiana* plants expressing plum pox virus coat protein. Plant Sci 91:157-169.

Ravelonandro, M., Monsion, M., Teycheney, P.Y., Delbos, R. and Dunez, J. 1992. Construction of a chimeric viral gene expressing *plum pox virus* coat protein: Gene 120:167-173.

Ravelonandro, M., Scorza, R., Bachelier, J.C., Labonne, G., Levy, L., Damsteegt, V., Callahan, A.M. and Dunez, J. 1997. Resistance of transgenic *Prunus domestica* to plum pox virus infection. Plant Dis 81:1231-1235.

Ravelonandro, M., Briard, P, Monsion, M., Scorza, R., Renaud, R. 2002°. 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 Hort 577: 91-96.

Ravelonandro, M., Scorza, R., Minoiu, N., Zagrai, I., and Platon, I. 2002b. Field tests of transgenic plums in Romania. Sanatatea Plantelor 60/2002:16-18.

Ravelonandro, M., Varveri, C., Delbos, R. and Dunez, J. 1988. Nucleotide sequence of the capsid protein gene of plum pox potyvirus. J Gen Virol 69:1509-1516.

Reveal, J.L. 2002. Checklist of the Vascular Plants of Maryland. http://www.inform.umd.edu/PBIO/pb250/mdfloramq.html

Rhoads, A.F. and Klein, W.M. 1993. The Vascular Flora of Pennsylvania: Annotated Checklist and Atlas. American Philosophical Society, Philadelphia, PA.

Rosales, M., Hinrichsen, P. and Herrera, G. 1998. Molecular characterization of plum pox virus isolated from apricots, plums and peaches in Chile. Acta Hort 472:401-405.

Rothstein, S.J., Jorgensen, R.A., Yin, J.C.-P., Yong-Di, Z., Johnson, R.C. and Reznikoff, W.S. 1981. Cold Spring Harbor Symp. Quant Biol. 45:99-105.

Sanford, J.C. and Johnston, S.A. 1985. The concept of parasite-derived resistance-deriving resistance genes from the parasite's own genome. J. Theor Biol. 113:395-405.

Scorza, R and H.W. Fogle. 1999. 'Bluebyrd' plum. HortScience 34:1129-1130.

Scorza, R., Callahan, A.M., Levy, L., Damsteegt, V. and Ravelonandro, M. 1998. Transferring potyvirus coat protein genes through hybridization of transgenic plants to produce plum pox virus resistant plums (*Prunus domestica* L.). Acta Hort. 472:421-427.

Scorza, R., Callahan, A., Levy, L., Damsteegt, V. and Ravelonandro, M. 2001. Resistance to plum pox potyvirus in a transgenic woody perennial fruit tree, European plum (*Prunus domestica* L.) results from post-transcriptional gene silencing. Acta Hort. 550:425-430.

Scorza, R., Callahan, A., Levy, L., Damsteegt, V., Webb, K. and 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. and Fogle, H.W. 1999. 'Bluebyrd' plum. HortScience 34:1129-1130.

Scorza, R., Levy, L., Damsteegt, V., Yepes, L.M., Cordts, J., Hadidi, A., Slightom, J.and Gonsalves, D. 1995. Transformation of plum with the papaya ringspot virus coat protein gene and reaction of transgenic plants to plum pox virus. J. Amer. Soc. Hort. Sci. 120:943-952.

Scorza, R., Ravelonandro, M., Callahan, A.M., Cordts, J., Fuchs, M., Dunez, J. and Gonsalves, D. 1994. Transgenic plums (Prunus domestica L.) express the plum pox virus coat protein gene. Plant Cell Repts 14:18-22.

Smith H.A., Powers H., Swaney S., Brown C., Dougherty W.G. 1995. Transgenic potato virus Y resistance in potato: evidence for an RNA-mediated cellular response. Phytopathology 85: 864-870.

Sonoda, S., Mori, M. and Nishiguchi, M. 1999. Homology-dependent virus resistance in transgenic plants with the coat protein gene of sweet potato feathery mottle potyvirus: Target specificity and transgene methylation. Phytopath. 89:385-391.

Stebbins, R.L. 1993. Growing prunes. Oregon State Univ. Extension Ser. EC 773. 6 p.

Strasburger, E., Noll, F., Schenck, H., Schimper, A.F. 1991. Lehrbuch der Botanik fur Hochschulen. Sitte P., Zeigler, H., Ehrendorfer, F., Bresinsky, A. (eds.) 33. Ed. Gustav Fischer Vg. Stuttgart, Jena, NY 778-780.

Takamatsu, N., Ishikawa, M., Meshi, T., and Okada, Y. 1987. Expression of bacterial chloramphenicol acetyl transferase gene in tobacco plants mediated by TMV-RNA. EMBO J. 6:307-311.

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

Varveri, C., Canderesse, T., Cugusi, M., Ravelonandro, M.M. and Dunez, J. 1988. Use of the 32P-labelled transcribed RNA probe for dot blot hybridization detection of Plum pox virus. Phytopathology 78:1280-1283.

Verwoerd, T.C., Dekker, B.M.M. and Hoekema, A. 1989. A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res. 17:2362.

Weinberger, J.H. 1975. Plums. pp. 336-347. In. Janick, J. and Moore, J.N. (eds) Advances in Fruit Breeding. Purdue University Press, West Lafayette IN.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M.B., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P., Green, A.G. and Waterhouse, P.M. 2001. Construct design for efficient, effective and high throughput gene silencing in plants. The Plant J. 27:581-590.

Wetzel, T., Candresse, T., Ravelonandro, M. and Dunez, J. 1991. A polymerase chain reaction assay adapted to plum pox potyvirus detection. J. Virol. Methods 33:355-365.

Wetzel, T., Candresse, T., Macquaire, G., Ravelonandro, M. and Dunez, J. 1992. A high sensitive immunocapture polymerase chain reaction method for plum pox virus detection. J. Virol. Methods 39:27-37.

Wilson, T.M.A. 1993. Strategy to protect crop plants against viruses: pathogen-derived resistance blossoms. Proc Natl Acad Sci USA 90:3134-3141.

Wypijewski, K., Malinowski, T., Musial, W., Augustyniak, J. 1994. The nucleotide sequence of coat protein gene of Polish isolate of plum pox virus (PPV-S). Acta Biochim. Pol. 41:87-95.

APPENDIX A

Biotechnology expands pest-management options for horticulture

Leonard Gianessi

Fruit and vegetable crops are under constant pressure from pests such as weeds, viruses, fungi, bacteria, insects and nematodes. If not controlled, many of these pests substantially lower yields. Successful agricultural production has depended on the use of pesticides for 100 years, and, yet, losses still occur due to certain pests that are poorly controlled. Some crops incur high costs for hiring laborers to hoe weeds because there are no effective herbicides. In addition, new pests routinely arrive for which effective controls have not yet been developed.

Agricultural researchers continuously seek out new methods to control pests, including biological agents, new chemicals and plant resistance through classical breeding. Biotechnology also offers a solution in some situations where traditional methods are ineffective or costly. Numerous researchers around the world are investigating biotechnological solutions to pest problems of horticultural crops. In 2002, the National Center for Food and Agricultural Policy released a study of current and potential biotechnological approaches to pest management in a wide array of crops (Gianessi et al. 2002).

Current plantings. The study identified three varieties of transgenic fruits and vegetables that are currently planted on small acreages in the United States: virus-resistant squash is grown on 5,000 acres in the Southeast, to prevent late-season losses to mosaic viruses; virus-resistant papaya is widely planted in Hawaii (2,000 acres)(see sidebar, page 92); and insect-resistant sweet corn is planted on a small number of acres and has reduced use of insecticide sprays.

Withdrawn varieties. Two transgenic horticultural varieties were available for a short time in the United States but were withdrawn due to marketing concerns. Insect- and virus-resistant New Leaf potatoes were planted on 4% of the nation's acreage in 1999 and were credited with reducing insecticide use. If the transgenic varieties had not been withdrawn due to processor resistance they could have been planted extensively in the Northwest, reducing insecticide use by 1.4 million pounds.

In 1999, the U.S. Environmental Protection Agency (EPA) granted Wisconsin sweet-corn growers emergency permission to spray herbicide-tolerant varieties (see sidebar, page 110). The transgenic varieties were not widely planted due to marketing concerns and growers have not reapplied for the use despite continued production losses.

If plum pox virus reaches California, the transgenic plum could help prevent losses to the state's multibillion dollar stone-fruit industry.

Crops currently being tested. Numerous fruits and vegetables have been transformed through genetic engineering and are being tested for their potential role in improving pest management. For example, University of Florida researchers are testing virus-resistant tomatoes as a substitute for the extensive insecticide spraying currently utilized to control insects vectoring geminiviruses. In California, herbicide-tolerant processing tomatoes have been tested and have the potential to reduce grower costs by $30 million and replace the use of 4.2 million pounds of fumigants.

Scott Bauer/USDA-ARS

Plums resistant to the plum pox virus have been developed by scientists with the U.S. Department of Agriculture but are not yet available to growers.

UC researchers have tested herbicide-tolerant lettuce that could reduce herbicide use by 140,000 pounds a year. Herbicide-tolerant strawberries could save Eastern growers several hundred dollars per acre in weed-control costs. Nematode-resistant pineapple is being developed at the University of Hawaii to replace 1.4 million pounds of fumigants. Insect-resistant broccoli developed at Cornell University could improve yields in years of heavy insect pressure. Virus-resistant raspberries developed by U.S. Department of Agriculture (USDA) researchers in the Northwest could help combat bushy dwarf virus, which is present in 80% of Northwest plantings. And transgenic apples resistant to fire blight bacteria have been developed and tested at Cornell University; the transgenic varieties would replace the use of antibiotics, which are used to kill the bacteria on 25% of U.S. apple acreage.

Emerging pests. Several research programs are focused on biotechnological approaches to control emerging pest problems. Plum pox virus was detected in the United States for the first time in Pennsylvania, where efforts are under way to eradicate it by destroying infected trees. USDA researchers have de-

veloped a virus-resistant plum that is being tested in Europe. If plum pox virus reaches California, the transgenic plum could help prevent losses to the state's multibillion dollar stonefruit industry.

Pierce's disease threatens California vineyards, and insecticide spraying has occurred to control the disease carrier, the glassy-winged sharpshooter. A researcher at the University of Florida (a state where Pierce's disease has been a problem for 80 years) has transformed grape tissue by inserting an antibacterial protein from another species into the grape genome. As a result, the transformed grape plant can destroy the bacteria without the need for insecticide sprays targeting the carrier.

Tristeza virus has killed 45 million citrus trees in Latin America and threatens the Texas citrus industry. Researchers at Texas A&M University have developed and are field testing virus-resistant trees.

Bacterial canker is present in Florida citrus orchards, and the state is trying to eradicate the disease by destroying infected trees, including millions of orchard and backyard citrus trees. A University of Florida researcher has developed and is testing a canker-resistant citrus tree.

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Reference

Gianessi LP, Silvers CS, Sankula S, Carpenter JE. 2002. Plant Biotechnology: Current and Potential Impact for Improving Pest Management in U.S. Agriculture; An Analysis of 40 Case Studies. National Center for Food and Agricultural Policy, Washington, DC. www.ncfap.org/40CaseStudies.htm.

Monsanto

Cotton has been genetically engineered to express a protein from a naturally occurring bacterium, *Bacillus thuringiensis*, which is toxic to insect pests such as bollworm and budworm. This cotton is widely planted in California and elsewhere in the United States.

when the leaf starts to senesce, leaf life is extended in transgenic plants exposed to drought, nutrition and pathogen stress (Gan and Amasino 1995; Clark et al. 2004).

Ethylene sensitivity. As in fruit ripening, manipulation of ethylene synthesis or sensitivity has applications in the ornamental plant industry. Ethylene accelerates floral and foliar senescence, and chemical methods have been developed to mitigate its effects (Sisler and Serek 2003). Ethylene sensitivity can be reduced in floriculture crops through applications of the ethylene antagonist silver thiosulfate (STS), but unfavorable environmental aspects such as metal contamination of groundwater restrict its commercial use. Another compound, 1-methylcyclopropene, also blocks the ethylene receptor protein and makes plant tissues insensitive to ethylene, delaying ripening or senescence. Although this compound is effective in many crops, its action decreases with time after treatment as the tissues synthesize new ethylene receptor proteins during postharvest transit. By expressing a mutant form of the ethylene receptor protein or by blocking expression of components of the ethylene-signaling pathway, petunia plants with longer lasting floral displays have been produced (Wilkinson et al. 1997). Unfortunately, negative side effects, such as higher susceptibility to fungal pathogens and decreased rooting of vegetative cuttings, have limited the commercial use of these technologies. The key to effective manipulation of ethylene sensitivity will be the use of promoters limiting transgene expression to the target tissue, leading ultimately to plants that have longer lasting flowers with no negative side effects.

Hurdles to commercialization

The lag in commercialization of transgenic horticultural crops clearly is not due to a lack of useful genes or valuable applications. However, several fundamental issues inherent to horticultural crops create significant hurdles (see sidebar, page 84).

Biological diversity. Simply the diversity of crops utilized in horticulture slows the adoption of new technologies. For any given crop, there may be several different species and dozens of cultivars that are currently marketed, and the turnover of new cultivars from year to year is tremendous. For example, as many as 60 distinct cultivars of iceberg lettuce alone may be grown throughout the year as production locations shift seasonally. Add to this the dozens of additional varieties for romaine, leafy, red and other specialty types, and it is evident that introducing a new biotech trait for lettuce requires developing not just one but many new varieties. In perennials such as trees and vines, on the other hand, the choice of a variety is a long-term

APPENDIX B

Plant Biotechnology: Current and Potential Impact For Improving Pest Management In U.S. Agriculture An Analysis of 40 Case Studies

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Viral Resistant Stone Fruit

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15. STONE FRUIT

Viral Resistant

Production

In 2000, there were 5,500 bearing peach tree acres in Pennsylvania. Total production was 60 million pounds valued at $17 million [1]. In addition to peaches, Pennsylvania orchardists maintain 868 acres of other stone fruits: 669 acres of nectarines, 160 acres of plums, and 39 acres of apricots [2]. Approximately one-half of Pennsylvania's peach tree acreage is located in Adams County on the southern border with Maryland [2].

Plum Pox Virus

In the summer of 1999, Adams County was the location of the first detection of plum pox virus in North America [17]. Plum pox virus (PPV), also widely known by its Bulgarian name, sharka, is a devastating disease of stone fruits, including plums, peaches, nectarines and apricots. It causes significant economic losses and is a limiting factor in stone fruit production in regions where it is found, including in several European countries. The disease first was reported in 1915 in Bulgaria and reached Yugoslavia in 1935 and Hungary about 1941. It spread more rapidly after 1950, reaching Germany in 1956, Poland and Romania in 1961, and France in 1970. The disease was eradicated in England in the 1960's only to reappear in the early 1970's. In 1984, Spain was invaded by plum pox [9]. Altogether, it has been estimated that over 100 million European trees are infected. Reported annual losses in Bulgaria have been as high as 60,000 tons of fruit. Reported yield losses in Poland have been greater than 50% and in Czechoslovakia they have averaged as high as 83.4% [4]. In Chile, plum pox symptoms first were detected in 1992. Fifteen percent of Chile's stone fruit acreage is infected.

Growers in Europe have learned to live with the disease [10]. The spread of plum pox is reduced by planting only certified disease-free plants, maintaining tight aphid spray programs and eliminating weeds that can be infected with the virus. A major control method is to remove trees showing symptoms immediately. In some cases, whole orchards have been removed [10].

As a result of the discovery of PPV in Adams County, PA, the Pennsylvania Agriculture Department established a quarantine of the affected areas. Canada immediately suspended all

import permits for plant material from the entire U.S. [3, 5], but has recently limited the ban to imports from Pennsylvania [6]. The Pennsylvania quarantine prohibits the movement of susceptible trees and propagative material within and out of the quarantine area. In March of 2000, the U.S. Secretary of Agriculture declared that PPV presented an emergency that threatened the nation's $2 billion stone fruit industry, and authorized the use of available funds for control and prevention of PPV spread, and eradication of PPV where it exists in the U.S. [7]. The USDA concluded that if steps were not taken to eradicate plum pox in Pennsylvania there was every possibility that the disease eventually would spread to other areas in the U.S. [7]. Therefore the USDA established: (1) a regulatory program to prevent the movement of plant material within or out of quarantine areas in Pennsylvania,; (2) a survey program to detect any additional infections; and (3) a control program to remove all infected orchards [7]. In January of 2001, the Pennsylvania Agriculture Department declared a moratorium on planting PPV-susceptible trees or shrubs in the PPV quarantine zones [8]. To date, the state has invested an estimated $5.1 million in PPV eradication and farmer indemnity, and approximately 1350 commercial acres of infected and exposed trees have been destroyed.

The Commonwealth of Pennsylvania has agreed to pay $1,000 an acre to growers to cover the cost of removing trees. Federal agencies approved a measure of $18 million to compensate growers for losses from plum pox, and the Pennsylvania Legislature approved $3 million in compensation.

Plum pox virus produces symptoms such as leaf discoloration and rings or spots on fruit [9]. Symptoms vary with host cultivar, plums being most severely affected. Symptoms include premature fruit drop, deformed fruit, and discoloration of the skin and flesh. Regardless of how severe leaf and fruit symptoms are, trees infected with plum pox will suffer from decreased production and reduced fruit quality with crop losses as high as 80 to 100% [3]. Much of the infected fruit drops prematurely, 20 to 30 days before normal maturity date. The fruit that remain on the trees lacks flavor and is low in sugar content. The virus so seriously affects the fruit of diseased trees that the fruit become unsuitable for direct consumption or industrial processing (dried, jams or brandied). Plum pox does not kill trees, but it makes the fruit unmarketable and drastically reduces yields.

Plum pox virus is spread over short distances, such as within and between orchards, by aphids. In Pennsylvania, one of the most efficient vectors is the green peach aphid. Aphids transmit PPV in a nonpersistent manner, which means uptake of the virus by the aphid and transmission to another plant occur within a few minutes.

The virus can occur in high concentrations in leaf cells, and when an aphid probes into an infected cell, some of the virus is sucked into the aphid's mouth parts, where it can stick to the lining of the food canal and remain infectious there for several minutes or hours. If the aphid then feeds on a healthy plant and continues to probe into a healthy epidermal cell, the virus carried in the food canal can detach and be squirted back into the healthy plant cell [9]. Research has determined that there are roughly 3.5 million aphids in a typical planted orchard acre, and between 50,000 and 300,000 aphids from that acre can visit a fruit tree in a one year period [3].

Long distance spread of PPV between geographic regions, and the method by which it is introduced into an area, is through transport of infected plants or plant parts, such as buds for grafting, by humans.

There is no treatment to cure virus infected trees, and once a tree is infected it serves as a source for infection of other trees. Identification, speedy removal and destruction of infected and potentially exposed trees is the only method of preventing increased spread of the virus. Combined with quarantine efforts to prevent renewed introduction of virus infected plant material to the area, these practices aim to at least contain, and at best eradicate, PPV infestations.

Detection of PPV, and the subsequent removal of trees, is expensive. The cost of tree removal and destruction is estimated at $1,500 per acre [10]. With 1350 acres already removed, well over $1 million has been spent in removal alone. In addition to this and the cost of replanting, the grower has to wait six years before replanted trees reach full production. The USDA mandates that once an orchard has been removed due to PPV infection, it cannot be replanted to PPV susceptible plants for three years. Growers can not replant susceptible prunus until the

quarantine is lifted – the quarantine will not be lifted until three years of no new positive finds in the survey [18]. The average annual value of an acre of mature stone fruit in Adams County is $1,038. For fruit growers in Pennsylvania, the only alternative to stone fruit is apple. But the profit margin on apples is lower than that for peaches, and with the area already so inundated with apple orchards, flooding the market with more would further reduce prices [3, 10].

Control of aphid populations with insecticides may reduce their levels, but because it takes only one aphid a few seconds to transmit PPV from an infected tree to a healthy one, insecticide applications will not effectively control PPV infestations. In the case of PPV, control through aphid management would be particularly difficult because there are several species of aphids that may transmit the virus. Scouting and treating for PPV-transmitting aphids would be necessary throughout the year, costing growers in time, labor and materials for a control strategy that is not adequately effective.

If eradication efforts are not successful in Pennsylvania, PPV threatens to spread to and devastate stone fruit production throughout North America. It has already been detected in Canada, and monitoring and grower education efforts are underway in other stone fruit production areas such as California [11]. The next line of defense is to plant varieties with resistance to PPV. Trees with PPV tolerance, although suffering few symptoms themselves, would still allow the virus to survive and replicate, serving as a source for continued spread. Only trees with resistance or immunity, whereby the virus cannot survive in the plant, will avoid losses and reduce incidences of PPV.

Viral Resistant Stone Fruit

For more than 50 years, classical plant breeding produced few stone fruit varieties that are highly resistant to PPV [14]. There are few sources for PPV resistance in stone fruit. Those that have been identified are controlled by multiple genes and are therefore very difficult to breed into new varieties.

The most recent progress in developing resistance to PPV has involved genetic engineering of resistant stone fruit species [9].

USDA ARS researchers in West Virginia, collaborating with researchers in Europe, have had success in engineering a transgenic plum variety with resistance to PPV [12]. Using *Agrobacterium*, they inserted viral coat protein genes into plum cells. The aim was to achieve coat protein mediated resistance, in which the ability of the plant cell to produce the viral coat protein somehow prevents invading viroids from uncoating, and thereby prevents them from replicating and spreading within the plant. Usually when this procedure is successful, high levels of viral coat genes and proteins are detected in the transformed plants. In one of the transformed plum lines, there was little detection of viral genetic material and no detection of viral or transgenic coat proteins. This transgenic plum line, C-5, exhibited excellent resistance to PPV whether inoculated through aphids or grafting using PPV infected budwood.

In greenhouse trials in which C-5 plum trees were exposed to aphids infected with PPV, and grafted with infected bud wood, the transgenic trees remained virus free for three years. In contrast, 30% of the non-transgenic trees in the experiment showed symptoms of the virus infection after six months, 67% showed symptoms after 12 months and 100% showed symptoms after 3 years [12].

The lack of viral protein in C-5 plants, and other characteristics, including the finding that the viral transgene insertion resulted in a complex transgene with multiple and aberrant gene copies, led researchers to conclude that the mechanism of PPV resistance in C-5 plums is due to post transcriptional gene silencing (PTGS), whereby the plant cell recognizes and shuts off invading viral genes, before they produce any proteins [13, 14]. Production of transgenic coat protein by the plant is also eliminated by PTGS.

The C-5 cultivar was produced using a new, commercially viable plum variety called 'Bluebyrd', which has excellent fruit quality as the female parent [16]. In addition, PPV resistance exhibited by C-5 transgenic plums is inherited in seed as a single gene, and is therefore available to incorporate into traditional plum breeding programs for future production of new varieties [14]. Ongoing work includes investigating the possibility of resistance expression in vegetative plant parts only so that the edible fruit will not be transgenic. Research also continues on developing a

successful transformation system for peaches, which so far has been elusive, so that the PPV resistance developed in C-5 plums can be incorporated into peach varieties [15].

The transgenic plum plants currently are restricted to laboratories in the U.S. Although the transgenic plum was developed in laboratories in the U.S., the trees are not being inoculated in the outdoors in the U.S. because of concerns about releasing the plum pox virus into the environment. Rather, the trees are being tested in orchard settings in Spain, France, Romania and Poland, where the virus is prevalent.

Estimated Impacts

It is assumed that a transgenic viral resistant stone fruit cultivar could be planted universally in Pennsylvania and would prevent the complete loss of production in the state (60 million pounds with a value of $17 million).

References

1. USDA, Noncitrus Fruits and Nuts 2000 Summary, National Agricultural Statistics Service, January, 2001.

2. USDA, 1997 Census of Agriculture – State Data, National Agricultural Statistics Service.

3. Wall, John, "A Plague Upon the Land", Penn State Agriculture, Summer/Fall 2000.

4. Canada Food Inspection Agency, "Pest Facts Sheet: Plum Pox Virus", available on the internet at www.cfia-acia.agr.ca/english/ppc/science/phra/plumpox/ppvfs_e.shtml.

5. Canada Food Inspection Agency, "Import Permits Suspended to Keep Out Plum Pox Virus", press release, November 22, 1999.

6. Brown, G., "Plum Pox Threat Prompts Changes in Nursery Industry", Fruit Growers News, April, 2001.

7. "Declaration of Emergency Because of Plum Pox Virus", Federal Register 65(42): 11280, March 2, 2000.

8. Pennsylvania Agriculture Department, "PA Agriculture Department Declares Moratorium on Planting in Plum Pox Virus Quarantine Area", press release, January 26, 2001.

9. Pennsylvania State University, "Plum Pox Virus of Stone Fruits", College of Agricultural Sciences, available on the internet at http://sharka.cas.psu.edu.

10. Welshans, J., et al., The Plum Pox Virus in Pennsylvania, Teaching and Learning Paper TLP 00-30, University of Florida, December, 2000.

11. "Growers Should Be Watchful for Powdery Mildew, Plum Pox' AgAlert, March 28, 2001.

12. Revalonandro, M., et al., "Resistance of Transgenic *Prunus domestica* to Plum Pox Virus Infection", Plant Disease, 81:1231-1235, November 1997.

13. Scorza, R., et al., "Post-Transcriptional Gene Silencing in Plum Pox Virus Resistant Transgenic European Plum Containing the Plum Pox Potyvirus Coat Protein Gene", Transgenic Research, in press.

14. Ravelonandro, M., et al., "The Use of Transgenic Fruit Trees as a Resistance Strategy for Virus Epidemics: The Plum Pox (Sharka) Model", Virus Research 71:63-69, 2000.

15. Scorza, R., USDA, ARS Appalachian Fruit Research Station, Kearneysville, WV. Personal Communication, April, 2001.

16. Callahan, Ann, USDA, ARS Appalachian Fruit Research Station, Kearneysville, WV. Personal Communication, April, 2001.

17. Levy, L., et al, "First Report of Plum Pox Virus (Sharka disease) in *Prunus persica* in the United States," Plant Disease, February 2000.

18. Halbrendt, John M., Pennsylvania State University, personal communication.