USDA/APHIS Environmental Assessment

In response to USDA-ARS Petition 04-264-01P seeking a Determination of Non-regulated Status for C5 Plum Resistant to Plum Pox Virus

OECD Unique Identifier ARS-PLMC5-6

U.S. Department of Agriculture
Animal and Plant Health Inspection Service
Biotechnology Regulatory Services

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I. Summary

The Animal and Plant Health Inspection Service of the United States Department of Agriculture (USDA-APHIS), has prepared an Environmental Assessment (EA) in response to a petition (APHIS Number 04-264-01p) from USDA, Agricultural Research Service (USDA-ARS) for a determination of non-regulated status for genetically engineered (transformed) ARS-PLMC5-6 plum (*Prunus domestica* L.) derived from their transformation event C5 (referred to hereafter as C5 plum). The genetically engineered C5 'Honeysweet' plum (*Prunus domestica)* was developed to resist infection by plum pox virus (PPV). This C5 plum is currently a regulated article under USDA regulations at 7 CFR Part 340, and as such, interstate movements, importations, and field tests of C5 plum have been conducted under a permit issued by APHIS (Permit #95-205-02r). USDA-ARS petitioned APHIS requesting a determination that C5 plum does not present a plant pest risk, and therefore C5 plum and its progeny derived from crosses with other non-regulated plum should no longer be regulated articles under these APHIS regulations.

II. Introduction

Plum pox (also referred to as Sharka disease) is the most devastating virus disease in *Prunus* species and considered an invasive species in the U.S. C5 plum was developed by using genetic engineering techniques to introduce the plum pox virus (PPV) coat protein (CP) gene into plum trees. Incorporation of the PPV-CP gene into the plum via *Agrobacterium*-mediated transformation does not cause plant disease, but rather enables C5 plum to resist infection by PPV. The PPV-CP gene was introduced into the plum as part of genetic construct that also included two plant-expressible genetic marker genes, *nptII and uidA (gus)*. These marker genes enable researchers to easily select those plant tissues that have been successfully transformed with the genetic construct.

PPV coat protein gene expression in C5 plum is under the control of the cauliflower mosaic virus (CaMV) 35S promoter, however, expression of the PPV coat protein gene in C5 plum does not result in production of PPV coat protein. The DNA regulatory sequences derived from the plant pathogens *Agrobacterium tumefaciens* and CaMV cannot cause plant disease by themselves or in conjunction with the genes that they regulate in the C5 plum.

Analysis of the C5 plum shows that it exhibits the characteristics of resistance based upon gene silencing. Multiple years of field trials of C5 and other transgenic plum events have been conducted in both the U.S. and Europe. These field trials have provided evidence that C5 plum resistance to plum pox disease is both effective against the major serotypes of PPV and stable under field conditions.

In accordance with APHIS procedures for implementing the National Environmental Policy Act (NEPA) (7 CFR Part 372), this EA has been prepared for C5 plum in order to specifically address the potential for impact to the human environment through the unconfined cultivation and use in agriculture of the regulated article.

A. USDA regulatory authority

APHIS regulations at 7 CFR Part 340, which were promulgated pursuant to authority granted by the Plant Protection Act (7 U.S.C. 7701-7772), regulate the introduction (importation, interstate movement, or release into the environment) of certain genetically engineered organisms and products. An organism is no longer subject to the regulatory requirements of 7 CFR Part 340 when it is demonstrated not to present a plant pest risk. A genetically engineered organism is considered a regulated article if the donor organism, recipient organism, vector or vector agent used in engineering the organism belongs to one of the taxa listed in the regulation and is also a plant pest, or if there is reason to believe that it is a plant pest. These plum trees have been considered regulated articles because they were genetically engineered with regulatory sequences and a viral coat protein gene derived from plant pathogens.

Section 340.6 of the regulations, entitled "Petition for Determination of Nonregulated Status", provides that a person may petition the Agency to evaluate submitted data and determine that a particular regulated article does not present a plant pest risk, and therefore should no longer be regulated. If APHIS determines that the regulated article is unlikely to present a greater plant pest risk than the unmodified organism, the Agency can grant the petition in whole or in part. In such a case, APHIS authorizations (i.e., permits or notifications) would no longer be required for field testing, importation, or interstate movement of the non-regulated article or its progeny.

B. Food and Drug Administration (FDA) Regulatory Authority

The FDA policy statement concerning regulation of products derived from new plant varieties, including those genetically engineered, was published in the Federal Register on May 29, 1992, and appears at 57 FR 22984-23005. Under this policy, FDA uses what is termed a consultation process to ensure that human food and animal feed safety issues or other regulatory issues (e.g., labeling) are resolved prior to commercial distribution of bioengineered food. USDA-ARS has submitted a food and feed safety and nutritional assessment summary to FDA for the C5 plum.

III. PURPOSE and NEED

APHIS has prepared this EA before making a determination on the status of C5 plum as regulated articles under APHIS regulations. The developer of these plum trees, USDA-ARS, submitted a petition to USDA-APHIS requesting that APHIS make a determination that these plum trees shall no longer be considered regulated articles under 7 CFR Part 340. Under regulations in 7 CFR Part 340, APHIS is required to give a determination on the petition for nonregulated status.

This EA was prepared in compliance with the National Environmental Policy Act (NEPA) of 1969 as amended, (42 USC 4321 *et seq*.) and the pursuant implementing regulations (40 CFR 1500-1508; 7 CFR Part 1b; 7 CFR Part 372).

IV. ALTERNATIVES

A. No Action: Continuation as a Regulated Article

Under the Federal "no action" alternative, APHIS would deny the petition. Under this alternative, C5 plum trees would continue to be regulated articles under the regulations at 7 CFR Part 340. Permits issued or notifications acknowledged by APHIS would still be required for introductions of C5 plum trees. APHIS might choose this alternative if there were insufficient evidence to demonstrate the lack of plant pest risk from the unconfined cultivation of plum trees engineered to express the coat protein of PPV.

B. Determination that C5 plum trees are No Longer Regulated Articles, in Whole

Under this alternative, C5 plums would no longer be regulated articles under the regulations at 7 CFR Part 340. Permits issued or notifications acknowledged by APHIS would no longer be required for introductions of plum pox virus resistant plum derived from these events. APHIS might choose this alternative if there were sufficient evidence to demonstrate the lack of plant pest risk from the unconfined cultivation of plum trees engineered with the coat protein of PPV and associated genes.

C. Determination that C5 plums are No Longer Regulated Articles, in Part

The regulations at 7 CFR Part 340.6 (d) (3) (I) state that APHIS may "approve the petition in whole or in part." APHIS might approve a petition in part if this partial approval would mitigate a potential plant pest risk. APHIS has not identified any greater plant pest risk characteristics in this transformed plum variety than non-transformed plum varieties that would warrant deregulation in part of C5 plum.

D. Preferred Alternative

APHIS has chosen Alternative B as the preferred alternative. This is based upon the lack of plant pest characteristics in the C5 plum variety.

V. Affected Environment

Plum species (*Prunus domestica*) are found native throughout the Northern Hemisphere with descriptions of plum dating back 2000 years (OECD 2002). The OECD Consensus Document on *Prunus* species provides a thorough overview on the biology of plum.

Prunus domestica (European or common plum) is an apparent natural alloploid between *P. cerasifera* which is diploid and *P. spinosa* which is tetraploid (OECD 2002). Many *P. domestica* cultivars are self-incompatible and may be cross-incompatible or cross-compatible. Pollen spread normally occurs via insect vectors (e.g., bees). Pollen of

Prunus species is normally not spread by wind, and self-pollination normally requires mechanical intervention of insects (OECD 2002). Most cultivated *Prunus* species (e.g., peach, nectarine, etc.) are diploid and do not naturally hybridize with *P. domestica* which is hexaploid (OECD 2002). While the *Prunus* OECD Consensus Document reports that sterile hybrids are normally produced between peach (*P. persica*) and *P. domestica*, there are reports of successful crosses between apricot *(P. armeniaca)* and other plum groups with *P. domestica* (OECD 2002).

While it is physically possible, introgression between cultivated *Prunus sp.* and wild relatives has been rarely seen (OECD 2002). Escapes of cultivated *Prunus sp.* are frequently found in woods, pastures, and abandoned orchards, but intercrosses with really wild populations have very little chance as they are extremely different in morphology and adaptation. In other words, hybrids could only survive in a protected environment (OECD 2002). Gene transfer to naturalized *Prunus* species in the U.S. is limited because of ploidy differences (Table 3, page 18-19 of petition) and the limited success of interspecific hybrids produced through controlled breeding.

A. Plum Pox Virus and Pathogen Derived Resistance

Plant viruses are ubiquitous in the environment and represent a significant threat to global agriculture because of their ability to reduce the quality and, more importantly, the yield of food and fiber crops (Matthews 1991; AIBS 1995; Hadidi, Khetarpal et al. 1998; Pappu 1999; Gonsalves, Gonsalves et al. 2004). Plant virus diseases cause damage to fruits, leaves, seeds, flowers, stems, and roots of many important crop species (OECD 1996). Hundreds of plant viruses have been described, affecting a wide range of plants and trees (ICTV 2005). These viruses infect virtually every plant species, and under natural conditions, certain plant viruses are nearly always present on particular crop or weed hosts (OECD 1996; Waterhouse 2001). The severity of virus infection can vary depending upon location and from one growing season to the next (OECD 1996).

Despite some diversity in size, shape and host range, plant viruses are very simple organisms that have small genomes and contain a limited number of genes (Matthews 1991; OECD 1996; Goldbach, Bucher et al. 2003). Most viruses are composed of proteinaceous capsids that encapsidate either RNA or DNA genomes. Some capsids may also contain carbohydrates and lipids (Matthews 1991; OECD 1996; Goldbach, Bucher et al. 2003). This proteinaceous coat plays an important role in protecting the genetic material, as well as in insect vector specificity and virus movement inside plants (Callaway, Giesman-Cookmeyer et al. 2001; Culver 2002).

Most plant viruses are obligate parasites that move from plant to plant via vector-mediated transmission[1] (Matthews 1991; OECD 1996). Plant viruses can also be spread in a number of other ways, depending upon the virus type, including seed transmission,

[1] Vector-mediated transmission can include: insects (e.g., aphids and whiteflies), nematodes, mites, and fungi.

pollen transmission, and/or mechanical[2] transmission (Matthews 1991; OECD 1996). In some agricultural regions, some crop species cannot be grown effectively because of the persistent presence of infected plant populations and/or potential virus vectors (OECD 1996). In other areas around the world, chemical pesticide sprays are used to help control insect vectors, but while these pesticide sprays provide the only means of relief, they are both expensive and not very effective in controlling virus disease spread (OECD 1996).

1. Plum Pox Virus

Plum pox virus is the causal agent of plum pox or Sharka disease, which is the most serious viral disease of plum and other *Prunus* species including: peach, apricot, nectarine, sweet cherry and sour cherry (Dunez 1988; Lopez-Moya, Fernandez-Fernandez et al. 2000; Ravelonandro, Scorza et al. 2000; Moustafa, Badenes et al. 2001; Gianessi 2002; Manganaris, Economou et al. 2003). Two major strains, or subgroups (PPV-M and PPV-D), and two minor strains of PPV (PPV-EA and PPV-C) have been identified (Glasa 2005). The PPV-EA and PPV-C strains represent a geographically-limited isolate (Egypt) and an isolate that is naturally able to infect cherries, respectively (Glasa 2005). Glasa and Candresse also report that there may be both a third major and third minor subgroup of PPV (Glasa 2005).

Plum pox virus can be spread over short distances, such as from tree to tree or orchard to orchard, via several species of aphid vectors. Aphids transmit PPV in a non-persistent manner[3] and therefore can acquire the virus from an infected tree and transmit the virus to a healthy tree within only a few minutes (Matthews 1991; Scorza 1994; Isac, Preda et al. 1998; Kegler, Fuchs et al. 1998; Lopez-Moya, Fernandez-Fernandez et al. 2000; Gianessi 2002). This is especially important when one considers reports which estimate that between 50,000 and 300,000 aphids can visit a single fruit tree within a one year period (Gianessi 2002). The virus can also be transmitted over both short and long distances through infected propagative material (i.e., budwood), which represents the primary source of PPV inoculum (Scorza 1994; Isac, Preda et al. 1998; Kegler, Fuchs et al. 1998; Lopez-Moya, Fernandez-Fernandez et al. 2000; Gianessi 2002).

Infected trees exhibit leaf and fruit chlorosis, fruit deformation, premature fruit drop, and in co-infections with other *Prunus*-infecting viruses, tree decline (APSnet; Moustafa, Badenes et al. 2001; Gianessi 2002). Since the disease was originally reported in Bulgaria (Atanassov 1932; Gianessi 2002; ICTV 2005), the virus has spread throughout

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[2] Mechanical transmission can include: intentional transfer of infected plant sap or purified virus in solution; vegetative propagation; infected host tissue; or contaminated equipment.

[3] In non-persistent aphid transmission, the viruses are acquired rapidly from plants (i.e., seconds), maintained in the aphid stylet, and can only be transmitted for a very short period of time (usually minutes) Hull, R. (2004). Matthew's Plant Virology. San Diego, CA, Elsevier Acadamic Press.

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Europe, where it is considered to be the most serious disease affecting stone fruit production and has destroyed more than 100 million trees (Lopez-Moya, Fernandez-Fernandez et al. 2000; Ravelonandro, Scorza et al. 2000; USDA/APHIS 2000; Moustafa, Badenes et al. 2001). More recently, the virus has spread to and caused significant damage in Asia, South America and North America (Levy 2000; Thompson 2001; Boulila 2004). Other than eradication of infected trees, there are no measures available to treat a PPV infection. Once a tree becomes infected with PPV, it can serve as a reservoir for virus transmission to other trees. This could be especially important in cases where a tree is tolerant of PPV infection and is not removed because of a lack of PPV symptoms (Minoiu, Maxim et al. 1998; Gianessi 2002).

In the U.S., where PPV is considered an invasive species (Clinton 1999; USDA/APHIS 2006), PPV-D was first detected in 1999 in Adams County, Pennsylvania (USDA/APHIS 2000; USDA/APHIS 2004). Since that time, local (Pennsylvania Department of Agriculture) and federal (USDA-APHIS) identification, control and eradication efforts have shown the virus to be limited to about 1600 acres of trees in three counties in Pennsylvania (USDA/APHIS 2004). Despite the somewhat minimal geographical distribution of the disease in the U.S., eradication efforts have exceeded $40 million (USDA/APHIS 2000; USDA/APHIS 2004). In Canada, where the disease is more widespread, the Canadian government has instituted a new seven year plum pox eradication program that essentially renewed the original three year eradication program that began in 2000 (CFIA 2005). This new Canadian program began in April 2004, with an initial allocation of $85 million from the Canadian government for plum pox virus eradication.

Currently, plum pox disease prevention relies upon use of certified virus-free planting material in addition to quarantine and eradication of infected materials. Greater than 50 years of traditional breeding for disease resistance has had only limited success (Fuchs, Gruntzig et al. 1998; Hartmann 1998; Minoiu, Maxim et al. 1998; Paprstein and Karesova 1998; Ravelonandro, Scorza et al. 2000; Moustafa, Badenes et al. 2001; Gianessi 2002). In cases where resistance has been identified, the resistance is controlled by multiple genes, which makes it very difficult to breed into new varieties (Gianessi 2002). If disease develops, the only control measure is tree destruction. However, eradication is not always a simple task. PPV is known to infect more than 30 *Prunus* species, as well as other plant species, all of which could potentially serve as reservoirs of the virus making eradication of the virus extremely difficult(Kegler, Fuchs et al. 1998; Ravelonandro, Scorza et al. 2000; Moustafa, Badenes et al. 2001; Damsteegt 2004; Scorza 2005).

2. Pathogen Derived Resistance

In general, the tools available for plant virus disease control are limited, as is their effectiveness in most instances. In cases where plants are susceptible to viruses, common control or management strategies have relied upon ineffective conventional measures of disease control such as use of virus-free planting material, vector control, or eradication (Gooding 1985; Superak, Scully et al. 1993; Swiezynski 1994; OECD 1996; Khetarpal,

Maisonneuve et al. 1998). Unlike other agricultural pests (e.g. insects), there are no chemical control measures that can be used directly to prevent or control plant virus disease outbreaks(OECD 1996; Hadidi, Khetarpal et al. 1998; Pappu 1999).

As an alternative approach, the concept of pathogen-derived resistance (PDR) was described about two decades ago (Sanford and Johnston 1985; Grumet, Sanford et al. 1987). Pathogen-derived resistance is based upon the use of pathogen-derived genes to generate specific host resistance (Goldbach, Bucher et al. 2003). One form of PDR is cross-protection which was first identified in 1929 (McKinney 1929) and involves intentional inoculation of crop plants with a closely related mild virus strain (Gooding 1985; Fulton 1986; Sherwood 1987; Beachy 1999; Goregaoker, Eckhardt et al. 2000; Culver 2002; Abbas M. 2005). Prior infection with a protecting or mild strain of a virus can prevent or interfere with infection by a related, more severe strain of the virus (Gooding 1985; Fulton 1986; Sherwood 1987; Beachy 1999; Goregaoker, Eckhardt et al. 2000; Culver 2002; Abbas M. 2005).

The mechanisms for cross protection have been determined to be either RNA-based or protein-mediated. RNA-based cross protection likely results from a gene silencing (post transcriptional gene silencing – PTGS) mechanism that targets viral RNA for destruction (Angell and Baulcombe 1997; Jan, Pang et al. 1999; Goregaoker, Eckhardt et al. 2000; Savenkov and Valkonen 2001; Culver 2002; Lacomme, Hrubikova et al. 2003; Lu, Martin-Hernandez et al. 2003; Baulcombe 2004; Chang, Chen et al. 2005). Protein-mediated cross protection likely relies upon several different mechanisms, including interference (Sherwood 1987; Beachy 1999; Goregaoker, Eckhardt et al. 2000; Culver 2002). This interference relies upon the coat protein of the mild strain of a virus to properly associate with and block disassembly of a more virulent strain of a virus, thus preventing replication and hence infection by the more virulent strain of the virus (Culver 2002).

In recent years, much of the research and development for plant virus disease control has focused on development of transgenic virus resistant plants. Building upon the concept of PDR and mechanisms previously described for cross protection, genetic modifications of host plants and trees are made that allow for expression of viral genes or proteins. Plant expression of viral genes or proteins often acts to delay or prevent infection by the same or related viruses. This form of PDR was first accomplished in 1986 by Roger Beachy and colleagues (Abel, Nelson et al. 1986) in which tobacco plants engineered to express tobacco mosaic virus (TMV) coat protein were resistant to TMV infection.

Since the initial successful development of a virus resistant transgenic plant, numerous other virus resistant plants and trees have been developed and field tested (Tepfer 2002; ISB 2005). Over the past 15 plus years, nearly 900 virus resistant plants and trees have been authorized by USDA-APHIS for field testing in the U.S. Some of these crops have been deregulated by APHIS and grown commercially in the U.S., including plants that express viral coat protein genes (e.g., papaya ringspot virus resistant papaya and ZW-20 squash) or a replicase protein gene (potato leafroll luteovirus resistant potato) (EPA 1998; Gonsalves 1998; ISB 2005). Most of this virus resistance is based on the pathogen-

derived resistance, and most often using VCP or VCP gene expression as the basis for resistance (Tepfer 2002; ISB 2005).

In the early 1990's, several researchers expressed PPV coat protein in transgenic plants (mostly tobacco) to determine if expression of PPV coat protein would provide an effective tool to combat plum pox disease development (Ravelonandro, Monsion et al. 1992; Ravelonandro, Monsion et al. 1993; Wypijewski, Musiao et al. 1995). Based upon this and other previous experience with transgenic virus resistant plants, transgenic plum was developed by Scorza and colleagues (Scorza 1994). The mechanism for resistance in the C5 plum was determined to be RNA-based (PTGS) (Scorza, Callahan et al. 2001; Hily, Scorza et al. 2004; Hily, Scorza et al. 2005). C5 plum trees do not produce detectable PPV coat protein and have shown stable and effective resistance to each of the major serotypes of PPV in field tests that have been conducted in three European countries over the past eight years (Scorza, Callahan et al. 2001; Hily, Scorza et al. 2004; Hily, Scorza et al. 2005).

APHIS authorized the first field testing of these plum trees in 1995 and they have been field tested in the United States under APHIS authorization (APHIS Permit # 95-205-02r) in subsequent years. No virus inoculations were allowed for field trials because of the invasive nature of this virus. However, field testing performed in the three European countries (Spain, Poland, & Romania) under appropriate permits from each country, included virus challenge experiments. C5 plum and its progeny have been evaluated extensively to confirm stability and that they exhibit the desired agronomic characteristics and do not present a plant pest risk. Field tests have been conducted in agricultural settings under physical and reproductive confinement conditions.

V. POTENTIAL ENVIRONMENTAL IMPACTS

Potential impacts to be addressed in this EA are those that pertain to the use of C5 plum and its progeny in the absence of confinement.

1. Potential impacts from gene introgression[4] from C5 plum into its sexually compatible relatives.

In assessing the risk of gene introgression from C5 plum into its sexually compatible relatives, APHIS considered two primary issues: 1) the potential for gene flow and introgression; 2) the potential impact of introgression.

Despite the low likelihood of introgression into relatives of C5 plum, consideration was given to what potential impact introgression could have on the environment if it was to occur. In the case of C5 plums, the primary concern is that transgene introgression would result in a domesticated, wild or weedy relative of plum becoming invasive because its

[4] Introgression is the successful, stable incorporation of a gene from one organism into another as a result of hybridization.

acquired virus resistance (Tepfer 2002; Stewart, Halfhill et al. 2003; Fuchs, Chirco et al. 2004(a); Fuchs, Chirco et al. 2004(b)). To consider this potential risk, several aspects of virus and plant biology should be considered.

In general, gene flow from cultivated agricultural crops to domesticated, wild or weedy relatives has most likely occurred ever since the domestication of a particular crop, assuming sexually compatible species are present (Stewart, Halfhill et al. 2003; Fuchs, Chirco et al. 2004(a)). Gene flow also can occur between virus resistant transgenic crops and non-transgenic crops (Fuchs, Chirco et al. 2004(a)). What is not as well understood is how much gene flow from transgenic virus resistant plants to wild or weedy relative's results in introgression of the gene(s), and what ecological impact this introgression would have. Stewart et al (2003) and others, discuss the basic difference between gene flow, such as through pollen, and introgression of genes, as well as the frequency of introgression (NRC 2000; Stewart, Halfhill et al. 2003; Fuchs, Chirco et al. 2004(a)). Based upon currently available data, there have been a relatively low number of confirmed cases of introgression (Stewart, Halfhill et al. 2003; Fuchs, Chirco et al. 2004(a)).

Even if it was shown that gene flow and introgression could occur with C5 plum, there is no clear evidence that shows the introgression of a virus resistance transgene into a plum relative would be any different than introgression of a naturally-occurring virus resistance gene from a non-transgenic plum (Tepfer 2002; Fuchs, Chirco et al. 2004(a)). Further, there is no evidence that indicates that a weedy plant would become more competitive, if it gained virus resistance via gene flow from VCP-expressing plants (EPA 2004). This is because, as discussed earlier, plant viruses are obligate parasites, and because of this, total destruction of their plant hosts would lead to the extinction of that virus (EPA 2004). It is assumed that there is a certain level of tolerance by some hosts – probably wild and weedy hosts – that allow for persistence of the virus. In fact, many virus infections do not produce visible symptoms in weeds (Falk and Bruening 1994; EPA 2004). Because of this, there likely exists a number of wild or weedy plant species that contain resistance genes that allow these plants to survive virus infection and serve as reservoirs for the virus (Raybould, Maskell et al. 1999).

This is somewhat different than the relationship between cultivated crops and plant viruses. Most of the major crop species used in today's agriculture (e.g. soybean, rice, wheat, beans) have been subjected to intense artificial selection over centuries and only have low survival under most natural conditions. The vast majority of the crops used in agriculture are much less fit, under natural conditions, than wild or weedy plants. Because of this, the impact of virus infection is potentially more severe than with some wild or weedy plants.

Finally, as mentioned earlier, PPV is an invasive species in the U.S. and has been the focus of significant eradication efforts. These eradication efforts, while successful in Pennsylvania, have been very expensive and were conducted on a relatively small scale – the efforts only involved three counties in Pennsylvania (USDA/APHIS 2000; USDA/APHIS 2004). Eradication efforts in Canada have been much more complicated because of the more widespread occurrence of the disease (Canadian Food Inspection

Agency 2005). Similar difficulties have also been encountered in other parts of the world where PPV is present. Therefore, even though it is very unlikely that gene flow and introgression of the PPV-CP resistance gene into plum relatives will occur, the net impact of introgression could be positive. If related species were resistant to the virus, thereby producing a critical tool in disease control by reducing potential reservoirs of the virus. Based on this, choosing Alternative B, granting non-regulated status may decrease the overall incidence of plum pox infection in cultivated and wild plants.

If APHIS chooses the no action alternative (Alternative A), APHIS would continue to regulate the environmental release of this resistant plum. There would be fewer plum pox resistant trees in the environment. The potential reduction in the plum pox reservoir would not occur. When plum pox re-enters the U.S. the resulting impact will be unchanged from its current state.

2. Potential impacts based on the relative weediness of C5 plum

P. domestica is not described as a weedy species and none of the *Prunus* species that may be sexually compatible with *P. domestica* are described as weedy species. In addition, plum is not listed as a Federal noxious weed or on other weed lists such as: Federal Noxious Weed List (http://www.aphis.usda.gov/ppq/weeds/noxwdsa.html), Washington State Weed Lists (http://www.nwcb.wa.gov/weed_list/weed_listhome.html), California Weed Species Lists (http://www.extendinc.com/weedfreefeed/list-b.htm), Montana County Noxious Weed List (http://www.weedawareness.org/weed%20list.html), North Dakota Noxious Weeds (http://www.ext.nodak.edu/extpubs/plantsci/weeds/w1103w.htm).

Because *P. domestica* is not described as a weedy species and none of the *Prunus* species that may be sexually compatible with *P. domestica* are described as weedy species, there would be no weed impact from deregulating this variety (Alternative B). If APHIS chooses the no action alternative (Alternative A) there would also be no weed impact from this variety.

3. Potential impact on non-target organisms, including beneficial organisms and threatened or endangered species

APHIS evaluated the potential for deleterious effects or significant impacts on non-target organisms, including those on the Federal Threatened and Endangered Species (TES) list of the U.S. Fish and Wildlife Service (FWS) (http://endangered.fws.gov/wildlife.html#Species), from cultivation of C5 plum and its progeny. An analysis was performed to determine if there were changes to insect fauna associated with trees expressing the PPV-CP or marker genes associated with the C5 plum. Data presented in Table 8 (page 66 of the petition) indicates that there was no correlation between insect damage and the transgenic or non-transgenic plum trees used.

The C5 plum does not express detectable coat protein from PPV, which eliminates concern of protein exposure to non-target organisms. Even if C5 did express viral coat

protein, however, this would not increase the issue of potential impacts to non-target organisms as the PPV coat protein is not known to have any toxic properties. In fact, viral coat proteins are routinely ingested by virtually all mammals when virus-infected fruits and vegetables are consumed. In addition, plant viruses are ubiquitous in the environment and cause damage to fruits, leaves, seeds, flowers, stems, and roots of many important crop species (Matthews 1991; AIBS 1995; Hadidi, Khetarpal et al. 1998; Pappu 1999; Gonsalves, Gonsalves et al. 2004). Hundreds of plant viruses have been described, affecting a wide range of plants and trees (ICTV 2005). These viruses infect virtually every plant species, and under natural conditions, certain plant viruses are nearly always present on particular crop or weed hosts (OECD 1996; Waterhouse 2001). The siRNAs present in C5 plum are also not of concern. Nucleic acids are a normal part of every living organism and do not have toxic or allergenic properties. Because of plant virus specificity, and because of the lack of protein production, the likelihood of impact on non-target organisms is virtually non-existent.

The *nptII* and *ß*-glucuronidase genes are commonly used marker genes found in soil-inhabiting *E. coli* bacteria. These bacteria are not plant or human pathogens, and do not cause disease symptoms or the production of infectious agents in plants. In addition, these marker genes are not known to cause adverse effects to non-target organisms and both have been granted exemption from the requirement of a tolerance by EPA for use in or on all raw agricultural commodities (EPA 1994; EPA 2001).
Analysis of both qualitative and quantitative information from the petition and published data, supports the developers conclusion that the unconfined release of C5 plum and its progeny would not harm any non-target or Federally listed (or proposed) threatened or endangered species. Consistent with APHIS' U.S. Fish and Wildlife Service TES assessment requirements, this is a "no harm" decision.

BRS has reviewed the data in accordance with a process mutually agreed upon with the U.S. Fish and Wildlife Service to determine when a consultation is needed as required under Section 7 of the Endangered Species Act." APHIS reached a determination that the release of C5 plums would have no effects to listed species and consequently a written concurrence or formal consultation with Fish and Wild Life Service is not required for this EA.

If APHIS chooses the no action alternative there would also be no impact on nontarget organisms or Federally listed endangered species.

4. Potential impacts on biodiversity

Analysis of available information indicates that C5 plum exhibits no traits that would cause increased weediness, that its unconfined cultivation should not lead to increased weediness of other cultivated plum or other sexually compatible relatives, and it is unlikely to harm non-target organisms common to the agricultural ecosystem or threatened or endangered species recognized by the U.S. Fish and Wildlife Service. Based on this analysis, there is no apparent potential for significant impact to biodiversity. If APHIS chooses the no action alternative there would also be no impact on biodiversity.

5. Potential for viral interactions and development of new viruses

APHIS has considered the physical and biological properties of PPV and its interactions with both its insect vectors and its host plants. PPV is considered to be an invasive species in the U.S. (Clinton 1999; USDA/APHIS 2006) and has been the focus of an eradication program since it was first detected in the U.S. in 1999 (USDA/APHIS 2000; USDA/APHIS 2004). While PPV is not currently present in the U.S., the aphid vectors for PPV are widely prevalent in the U.S. in areas where plums are grown.

1. Heterologous Encapsidation

Heterologous encapsidation occurs when the coat protein of one virus is able to encapsidate the nucleic acid of a second virus. Heterologous encapsidation was first described by Rochow (1970) and has been the subject of numerous reviews (Rochow 1977; Falk and Duffus 1981; Falk, Passmore et al. 1995; Miller, Koev et al. 1997; Tepfer 2002). In some cases, these two or more viruses may be related, while in other scenarios, the viruses may be completely unrelated (Falk, Passmore et al. 1995; Tepfer 2002). The majority of heterologous encapsidation interactions that have been identified involve luteoviruses (Rochow 1977; Falk, Passmore et al. 1995; Miller, Koev et al. 1997). These interactions occur naturally in both agricultural crop and weed plants, and are a natural part of virus-virus and virus-plant interactions (Rochow 1977; Falk and Duffus 1981; Falk, Passmore et al. 1995). In some cases, heterologous encapsidation is a specific interaction between two viruses that plays an important role in both virus biology and survival (such as in the case of helper-dependent transmission[5]) (Falk, Passmore et al. 1995).

In the case of C5 plum, the potential for heterologous encapsidation is essentially non-existent. Data on the C5 plum shows that the mechanism of resistance is based upon PTGS. Therefore, because it appears that no PPV coat protein is produced in these trees, there is essentially no potential for C5 plum expressed PPV-CP encapsidating RNA from other plant viruses.

2. Recombination

It is theoretically possible for new plant viruses to arise in the C5 plum through recombination and APHIS has considered this issue in its evaluation of this petition. Recombination is defined as the exchange of nucleotide sequences between two nucleic acid molecules (USDA/APHIS 1996; USDA/APHIS 1999). Recombination between viral genomes can result in heritable, permanent change (USDA/APHIS 1996; USDA/APHIS 1999). The persistence of the recombined viral genome depends upon its fitness with respect to its ability to replicate within the original host cell, its ability to replicate in the presence of the parental viruses, its ability to spread systemically within the host, and its successful transmission to other host plants.

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[6] Helper-dependent transmission often involves a virus that lacks a coat protein becoming encapsidated into the coat protein of another virus allowing for subsequent insect transmission of the coat protein-lacking virus.

Recombination events in plant viruses contribute to evolution of the viral genome (Falk and Bruening 1994; Gibbs and Cooper 1995; Roossinck 1997; Aaziz and Tepfer 1999; Rubio, Borja et al. 1999; Worobey and Holmes 1999; Tepfer 2002). RNA-RNA recombination occurs between closely related RNA molecules, but also between dissimilar RNAs, possibly at sites of similar RNA structure (Falk and Bruening 1994; Roossinck 1997).

Under normal agricultural conditions, plant viruses have numerous opportunities to interact genetically (Falk and Bruening 1994). Multiple or mixed infections, where more than one virus infects a crop or weed host, are common in nature. Some reports have shown five or more different viruses infecting the same plant (Falk and Bruening 1994; Falk, Passmore et al. 1995; EPA 2004). Falk and Bruening suggest that these mixed infections probably occur more frequently than what has been reported, and because most plant viruses can infect most plant protoplasts, suggesting their potential to infect individual plant cells, mixed subliminal and conventional infections have likely already brought together numerous combinations of virus genes (Falk and Bruening 1994). Therefore, under natural field conditions, it is possible for viruses that cannot systemically infect a particular plant to interact with viruses that are capable of systemic infection (Falk and Bruening 1994). Although there is potential for these viruses to continuously interact under natural settings, new viral diseases are normally due to minor variants of existing viruses as opposed to new viruses resulting from recombination (Falk and Bruening 1994). The idea of new variants arising from existing viruses, and being responsible for virus diseases is strongly supported by the level of variability that occurs within individual viruses (Falk and Bruening 1994; Gibbs and Cooper 1995; Roossinck 1997; Aaziz and Tepfer 1999; Rubio, Borja et al. 1999; Worobey and Holmes 1999; Tepfer 2002).

According to Bruening (2000), it is highly unlikely given the high background of recombination known to occur naturally in mixed infections of both crop and wild plants that the risk of recombination would be any different in transgenic plants (Bruening 2000). Most scientific literature suggests that such an event would be a rare occurrence (Falk and Bruening 1994; USDA/APHIS 1999; EPA 2004). In further considering this issue, one must also consider what risk such a recombination event would pose. Given that recombination is widely accepted as a significant part of virus evolution, and that multiple viruses are commonly found in a single plant providing ample opportunity for interaction, the likelihood that transgenic VCP-expressing plants present a greater risk to the environment is low.

Plum pox virus is a member of the potyviridae which is a large group of RNA plant viruses that infect a wide range of plant species (Matthews 1991; ICTV 2005). Other than PPV, there have not been other reports of potyviruses infecting *Prunus* species (Matthews 1991; ICTV 2005). Therefore, while there have been reports of recombination between PPV strains (Glasa 2001; Glasa 2002), the likelihood of recombination between PPV-CP and other potyviruses in C5 plum trees is very low. Further, most of the viruses that occur in *Prunus* species in Europe also occur in the U.S., and there have not been reports of recombination events between PPV and other viruses in Europe under natural conditions and where the C5 trees have been tested. Based upon

what we know about the biology of plant viruses, and data that we have gathered from Europe, the likelihood of recombination events between the C-5 plum expressed PPV-CP and other plant viruses is very low.

3. Synergy

Synergy occurs when two independent viruses infect a plant simultaneously and the resulting disease symptoms are more severe than when either virus infects the plant individually (Matthews 1991; OECD 1996; Pruss, Ge et al. 1997; Tepfer 2002). Synergistic infections typically result in agronomic problems, producing diseased, unmarketable crops, rather than environmental impacts. Their occurrence would not likely be any different in transgenic crops than in naturally mixed infections (USDA/APHIS 1996).

Several naturally-occurring synergistic virus interactions have been described, with the majority of the combinations involving at least one potyvirus (Rochow and Ross 1955; Vance 1991; Vance, Berger et al. 1995; OECD 1996; Pruss, Ge et al. 1997; Tepfer 2002). Vance and colleagues have shown that when plants are co-infected by both a potyvirus (e.g., potato virus Y virus – PVY; tobacco vein mottling virus – TVMV; pepper mottle virus - PeMV) and potato virus X virus (PVX), the disease symptoms are significantly worse than plants infected with either of the viruses alone (Vance 1991; Vance, Berger et al. 1995). In addition to the change in disease symptoms, there was a significant increase in PVX virus particles without any corresponding increase in PVY virus particles (Vance 1991).

While there is potential for synergistic interactions to occur between PPV and other viruses, there is no evidence to suggest that potyviral coat protein genes alone are involved in synergy. Therefore, it is unlikely that use of C5 plum would increase the potential for synergistic interactions.

6. Potential impacts on commercial use

If APHIS takes no action, commercial scale production of C5 plum and its progeny is effectively precluded. These trees could still be grown under APHIS permit as they have been for the past several years. However, widespread, unconfined use of the trees would not be allowed as long as the C5 plum is considered a regulated article. APHIS has evaluated field trial data reports submitted on this event and progeny, and has noted no significant adverse effects on non-target organisms, no increase in fitness or weediness characteristics, and no effect on the health of other plants. The Agency expects that if these trees were grown under permit in the future, that they would perform similarly. If APHIS were to grant the petition for non-regulated status in whole, C5 plum and its progeny would no longer be considered regulated articles. The unrestricted cultivation and distribution of C5 plum would be allowed and would not subject to regulation by APHIS under 7 CFR Part 340.

From a commercial perspective, current methods for control of this virus are both ineffective and expensive. The USDA-APHIS began an eradication program in 2000 in an effort to remove PPV infected trees in three counties in Pennsylvania. While this

eradication program was successful, it was expensive, and was conducted on a relatively small scale as the virus had only spread to these three counties in Pennsylvania. Eradication efforts in Canada have been much more expensive and more complicated given the widespread nature of the virus. If C5 plum was no longer considered a regulated article (Alternative B), it could add a potentially more effective, cheaper and preemptive means of control of an invasive species in the U.S. The C5 plum trees could be grown on a large-scale basis without confinement restrictions that are imposed in release permits.

Therefore, if APHIS were to take no action (Alternative A), and growers do not have PPV resistant varieties of plum trees derived from C5 plum, they would likely have to rely upon cultural practices to reduce the potential impact of PPV. USDA-APHIS-Plant Protection and Quarantine (USDA/APHIS/PPQ) conducted an environmental assessment (EA) in 2000 to assess the potential impact of a PPV eradication program in Pennsylvania(USDA/APHIS 2000). In this EA, PPQ described the limited effectiveness of using cultural practices to control PPV and reached a determination that the adverse effects of selecting the no action alternative to PPV eradication could have significant environmental impact (USDA/APHIS 2000). Other than eradication and use of clean propagative material, there are no other effective control measures for plum pox. If the disease were to occur in the U.S. with wider geographical distribution than has been seen in Pennsylvania, and as has occurred in Canada and Europe, the disease could cause devastating losses to both commercial and private stone fruit trees in the U.S. As stated in the USDA plum pox eradication environmental assessment document (USDA/APHIS 2000), a widespread plum pox infestation could greatly reduce the supply of agricultural commodities and home produce.

Plum pox virus has been shown to have a host range that includes ornamental and wild *Prunus* species, some common weeds (clover and lamb's quarters) as well as some garden plants (tomatoes, petunias and zinnias) (USDA/APHIS 2000). These infected trees and plants could serve as hosts for the virus and reservoirs for further spread of the virus. Therefore, while the occurrence of the disease in the U.S. has been limited to date, there is significant potential for widespread impact on a much larger scale if the virus were to be re-introduced into the U.S in the future, which is likely given the close proximity of the disease in Southern Canada.

Field tests conducted over the past eight or so years have shown the C5 plum trees to be resistant to infection by PPV, even under conditions of high disease pressure. Further, the PPV resistance has been shown to be stable and inheritable. Despite the fact that the PPV-CP gene is derived from a plant pathogen, the coat protein gene itself cannot cause plant disease. The data provided in this petition indicate that the mechanism for resistance is based upon PTGS. Because of the lack of protein production, there would be no adverse effects from protein exposure and no potential for heterologous encapsidation. The potential for synergy and recombination would be low. While PPV is not currently present in the U.S., there is a tremendous amount of knowledge about potyviruses. In addition, most of the viruses related to PPV that occur in the U.S. also occur in Europe and other areas where PPV occurs, yet there have not been any reports of new or more pathogenic viruses/diseases developing from their interactions with PPV.

Finally, as discussed previously in this EA, gene transfer from C5 plum to naturalized *Prunus* species is limited because ploidy differences and the limited success of interspecific hybrids produced through controlled breeding.

7. Potential impacts on organic farming

The National Organic Program (NOP) administered by USDA's Agricultural Marketing Service (AMS) requires organic production operations to have distinct, defined boundaries and buffer zones to prevent unintended contact with prohibited substances from adjoining land that is not under organic management. Organic production operations must also develop and maintain an organic production system plan approved by their accredited certifying agent. This plan enables the production operation to achieve and document compliance with the National Organic Standards, including the prohibition on the use of excluded methods. Excluded methods include a variety of methods used to genetically modify organisms or influence their growth and development by means that are not possible under natural conditions or processes.

Organic certification involves oversight by an accredited certifying agent of the materials and practices used to produce or handle an organic agricultural product. This oversight includes an annual review of the certified operation's organic system plan and on-site inspections of the certified operation and its records. Although the National Organic Standards prohibit the use of excluded methods, they do not require testing of inputs or products for the presence of excluded methods.

The presence of a detectable residue of a product of excluded methods alone does not necessarily constitute a violation of the National Organic Standards. The unintentional presence of the products of excluded methods will not affect the status of an organic product or operation when the operation has not used excluded methods and has taken reasonable steps to avoid contact with the products of excluded methods as detailed in their approved organic system plan. Organic certification of a production or handling operation is a process claim, not a product claim.

It is not likely that organic farmers, or other farmers who choose not to plant transgenic varieties or sell transgenic plum, will be significantly impacted by the expected commercial use of this product since: (a) nontransgenic plum will likely still be sold and will be readily available to those who wish to plant it; (b) plum trees propagated by grafting and growers purchasing budwood or grafted plants will know that this product is transgenic because it will be marketed as plum pox virus resistant plum. Additionally, decreasing the overall incidence of plum pox in conventional orchards may lower the likelihood of an organic orchard becoming infected.

This particular product should not present new and different issues than those with respect to impacts on organic farmers. APHIS has considered that gene transfer to naturalized *Prunus* species in the U.S. is limited because of ploidy differences (Table 3, page 18-19 of petition), a lack of documented natural outcrossing and the limited success of interspecific hybrids produced through controlled breeding.

If APHIS chooses the no action alternative there would be no direct impact on organic farmers and the current cultivation practices are unlikely to change. However, in the absence of plum pox resistant plum, the opportunity for plum pox to establish in plum orchards is greater. This may provide more routes to infect organic orchards.

8. Potential impacts on raw or processed agricultural commodities

APHIS analysis of data on agronomic performance, disease and insect susceptibility, and compositional profiles of the plums indicate no significant differences between C5 plum and non-transgenic counterparts that would be expected to cause either a direct or indirect plant pest effect on any raw or processed plant commodity from deregulation of line C5. C5 plum is also undergoing review by the FDA for use in food and feed (http://www.cfsan.fda.gov).

VI. CONSIDERATION OF EXECUTIVE ORDERS, STANDARDS AND TREATIES RELATING TO ENVIRONMENTAL IMPACTS

Executive Order (EO) 12898, "Federal Actions to Address Environmental Justice in Minority Populations and Low-Income Populations," requires Federal agencies to conduct their programs, policies, and activities that substantially affect human health or the environment in a manner so as not to exclude persons and populations from participation in or benefiting from such programs. It also enforces existing statutes to prevent minority and low-income communities from being subjected to disproportionately high and adverse human health or environmental effects.
EO 13045, “Protection of Children from Environmental Health Risks and Safety Risks,” acknowledges that children may suffer disproportionately from environmental health and safety risks because of their developmental stage, greater metabolic activity levels, and behavior patterns, as compared to adults. The EO (to the extent permitted by law and consistent with the agency’s mission) requires each Federal agency to identify, assess, and address environmental health risks and safety risks that may disproportionately affect children. Each alternative was analyzed with respect to EO 12898 and 13045. None of the alternatives are expected to have a disproportionate adverse effect on minorities, low-income populations, or children.

EO 13112, “Invasive Species”, states that federal agencies take action to prevent the introduction of invasive species and provide for their control and to minimize the economic, ecological, and human health impacts that invasive species cause. Non-engineered plum is widely grown in the United States. Based on historical experience with these varieties and the data submitted by the applicant and reviewed by APHIS, the engineered plant is sufficiently similar in fitness characteristics to other plum varieties currently grown and it is not expected to have an increased invasive potential.

Introduction of C5 plum trees results in the introduction of a genetic portion of plum pox virus, which is considered an invasive species in the U.S. (USDA/APHIS 2002; USDA/APHIS 2006). However, the coat protein gene of PPV cannot itself cause disease. In addition, the PPV-CP gene expressed in C5 plum could provide a means of resistance

to the PPV which supports EO 13112 to "provide for their control and to minimize the economic, ecological…..impacts that invasive species cause".

Executive Order 12114, "Environmental Effects Abroad of Major Federal Actions" requires Federal officials to take into consideration any potential environmental effects outside the U.S., its territories and possessions that result from actions being taken. APHIS has given this due consideration and does not expect a significant environmental impact outside the United States should non-regulated status be determined for C5 plum or if one of the other alternatives is chosen. It should be noted that all the considerable, existing national and international regulatory authorities and phytosanitary regimes that currently apply to introductions of new plum cultivars internationally, apply equally to those covered by an APHIS determination of non-regulated status under 7 CFR Part 340. Any international traffic of C5 plum subsequent to a determination of non-regulated status for C5 plum would be fully subject to national phytosanitary requirements and be in accordance with phytosanitary standards developed under the International Plant Protection Convention (IPPC).

The purpose of the IPPC "is to secure a common and effective action to prevent the spread and introduction of pests of plants and plant products, and to promote appropriate measures for their control" (https://www.ippc.int/IPP/En/default.jsp). The protection it affords extends to natural flora and plant products and includes both direct and indirect damage by pests, including weeds. The IPPC has set a standard for the reciprocal acceptance of phytosanitary certification among the nations that have signed or acceded to the Convention (137 countries as of April 2005). In April, 2004, a standard for pest risk analysis (PRA) of living modified organisms (LMOs) was adopted at a meeting of the governing body of the IPPC as a supplement to an existing standard, International Standard for Phytosanitary Measure No. 11 (ISPM-11; Pest Risk Analysis for Quarantine Pests). The standard acknowledges that all LMOs will not present a pest risk, and that a determination needs to be made early in the PRA for importation as to whether the LMO poses a potential pest risk resulting from the genetic modification. APHIS pest risk assessment procedures for bioengineered organisms are consistent with the guidance developed under the IPPC. In addition, issues that may relate to commercialization and transboundary movement of particular agricultural commodities produced through biotechnology are being addressed in other international forums and through national regulations.

The Cartagena Protocol on Biosafety is a treaty under the United Nations Convention on Biological Diversity (CBD) that established a framework for the safe transboundary movement, with respect to the environment and biodiversity, of LMOs, which includes those modified through biotechnology. The Protocol came into force on September 11, 2003 and 132 countries are Parties to it as of March 6, 2006 (see http://www.biodiv.org/biosafety/default.aspx). Although the United States is not a party to the CBD, and thus not a party to the Cartagena Protocol on Biosafety, US exporters will still need to comply with domestic regulations that importing countries that are Parties to the Protocol have put in place to comply with their obligations. The first intentional transboundary movement of LMOs intended for environmental release (field

trials or commercial planting) will require consent from the importing country under an advanced informed agreement (AIA) provision, which includes a requirement for a risk assessment consistent with Annex III of the Protocol, and the required documentation. LMOs imported for food, feed or processing (FFP) are exempt from the AIA procedure, and are covered under Article 11 and Annex II of the Protocol. Under Article 11 Parties must post decisions to the Biosafety Clearinghouse database on domestic use of LMOs for FFP that may be subject to transboundary movement. To facilitate compliance with obligations to this protocol, the US Government has developed a website that provides the status of all regulatory reviews completed for different uses of bioengineered products (http://usbiotechreg.nbii.gov). This data will be available to the Biosafety Clearinghouse. APHIS continues to work toward harmonization of biosafety and biotechnology consensus documents, guidelines and regulations, including within the North American Plant Protection Organization (NAPPO), which includes Mexico, Canada, and the United States and in the Organization for Economic Cooperation and Development. NAPPO has completed three modules of a standard for the *Importation and Release into the Environment of Transgenic Plants in NAPPO Member Countries* (see http://www.nappo.org/Standards/Std-e.html). APHIS also participates in the North American Biotechnology Initiative (NABI), a forum for information exchange and cooperation on agricultural biotechnology issues for the U.S., Mexico and Canada. In addition, bilateral discussions on biotechnology regulatory issues are held regularly with other countries including: Argentina, Brazil, Japan, China, and Korea. Many countries, e.g. Argentina, Australia, Canada, China, Japan, Korea, Philippines, South Africa, Switzerland, the United Kingdom.

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Appendix A: Summary table of data submitted with petition 04-264-01p for C5 Plum

Appendix B: Summary of petition data and information considered in completing environmental assessment

1. Description of Transformation System:

The *Agrobacterium* transformation system used to develop C5 plum has been previously described by Mante et al. and Scorza et al. (Mante 1991; Scorza 1994). Transformation with *Agrobacterium* should not lead to crown gall disease in C5 plum because the *Agrobacterium tumefaciens* strain was disarmed by removing the native T-DNA from C58/Z707. The native T-DNA, which contains the plant hormone genes necessary for the formation of crown gall tumors, was replaced by the PPV-CP cassette. Further, antibiotics were used to kill any remaining *Agrobacterium* after transformation.

The C5 plum was transformed using the previously described binary plasmid pGA482GG (Fitch 1990; Ling 1991). This plasmid was also used in the previously deregulated papaya ringspot virus resistant papaya (APHIS, 1996). The pGA482GG plasmid contains the *nptII* and *uidA* (gus) marker genes, as well as tetracycline and gentamicin antibiotic resistance genes. The *nptII* gene is under control of the nopaline synthase promoter (nos) and nos terminator. The *uidA* gene is under control of the 35S promoter and nos terminator. The tetracycline and gentamicin marker genes are under control of prokaryotic promoters and therefore are not expressed in plants. In addition to these intact genes, pGA482GG contains an interrupted ß-lactamase gene. Sequencing analysis show that this gene is interrupted by a *cos* site that renders the gene non-functional.

The PPV-CP gene cassette, containing the 35S promoter, from plasmid pBIPCP (Ravelonandro, Monsion et al. 1992) was subcloned into *Hind*III-digested pGA482GG and the resulting plasmid was designated pGA482GG/PPV-CP-33 (see Figure 1, page 20 of Petition for schematic diagram of the PPV-CP cassette). This plasmid was used to electrotransform *Agrobacterium tumefaciens* strains C58/Z707. This is the same *A. tumefaciens* that was previously used in the deregulated papaya ringspot resistant papaya (USDA/APHIS 1996). The transformed *A. tumefaciens* was grown overnight at 28 °C in 10 ml Luria broth with 50 µg/ml kanamycin and 50 µg/ml gentamicin; centrifuged at 4000 x g for 10 min; resuspended in 10 ml bacterium resuspension medium[6] with 2% sucrose, 100 µM acetosyringone and 1 mM betaine phosphate; and shaken for 6 hr at 20° C before use.

2. Characterization of DNA inserted into C5 plum

A series of analyses were conducted to characterize the DNA inserted into C5 plum, including Southern blot analysis and DNA sequencing. Briefly, DNA was isolated from C5 plum, four other putatively transformed plums (C2-C4 & C6), and non-transformed 'Bluebyrd' plum. DNA was digested with restriction enzymes *Bam*HI and *Eco*RI. Southern blot analysis of *Bam*HI digested C5 DNA show the expected 1.2 kb fragment,

in addition to a second, larger fragment (> 2kb). The developers suggest that this larger than expected fragment likely resulted from a rearrangement. DNA signal intensity analysis suggests that the C5 contains between 1 and 4 copies of the PPV-CP gene (Figure 4, page 26 of petition). Southern blot analysis of EcoRI digested C5 DNA showed the expected 7 kb fragment, along with other larger and smaller fragments, which suggest multiple insertions of the PPV-CP gene (Figure 4, page 26 of petition).

Further analysis was performed to more fully characterize the PPV-CP insert in C5 plum. DNA from the C5 plum was digested with *Eco*RI, *Hind*III, and *Bam*HI and analyzed by Southern blot analysis using either the1 kb from the PPV-CP gene, the 1.1 kb fragment from the *nptII* gene, or the 0.8 kb fragment from the *uidA* gene as a probe. Figure 5 (page 27 of the petition) shows the results of the *Eco*RI digest. Each of the digestions showed that the full-length PPV-CP gene was incorporated into the C5 plum genome.

In addition to the Southern analysis of the PPV-CP insert, a bacterial artificial chromosome (BAC) library was developed from C5 plum and sequenced. Because of the complexity of the insert, including sequence repeats, DNA methylation and the bacterial plasmid origin of replication, sequencing results represent approximately 80% of the insert. The combination of this sequencing and the restriction analysis allowed for development of a schematic diagram of the components of the transgene inserted in C5 plum (Figure 6, page 30 of petition). In addition, the sequence analysis provided evidence that the ß-lactamase gene in C5 plum is interrupted by a fragment containing a bacterial *cos* site (Figure 1, page 20 of petition) and is therefore inactivated.

The *nptII* and *ß*-glucuronidase genes, commonly used as marker genes, are found in soil-inhabiting *E. coli*. These bacteria are not plant or human pathogens, and do not cause disease symptoms or the production of infectious agents in plants. The PPV-CP cassette contains the leader sequence from the TMV coat protein and an ATG start codon fused to the PPV coat protein gene from the PPV-D strain (Ravelonandro et al., 1992; Takamatsu et al., 1987). Both the TMV leader sequence and the PPV coat protein gene are components of naturally occurring plant viruses, but neither of these genes is capable of causing plant or human disease. The commonly used 35S promoter is derived from cauliflower mosaic virus which is a plant pathogen. Cauliflower mosaic virus (CaMV) causes disease primarily in cruciferous plants. However, the CaMV 35S promoter does not cause disease symptoms in plants, nor does it encode for an infectious agent.

3. RNA and Protein Characterization and Expression:

Northern blot analysis was performed on each of the five transformed plum lines (C2 - C6) and a non-transformed control plant (‘Bluebyrd’). Figure 2 (page 23 of the petition) shows the expected 1.4 kb transcript present in each line, as well as the relative amounts of PPV-CP RNA found in each line. These results show that the amount of transcript RNA present in C5 plum was much less than that found in C2-C4 plum. These results are consistent with those previously described by Scorza, et al (Scorza 1994). As expected, no transcript RNA was found in the non-transformed control.

Western blot analysis was used to analyze protein production in each of the five transformed lines (C2-C6). Figure 3, page 24 of the petition shows the results of the immunoblot that was performed with monoclonal antibodies raised against the PPV coat protein. Results of this testing showed protein production in transformed lines C2-C4, but no detectable protein produced in the C5 and C6 lines. This lack of detectable protein is consistent with the lack of protein produced in C5 plum field trials, as well as the suggested mode of virus resistance based upon gene silencing (Scorza, Callahan et al. 2001; Scorza 2005).

4. Mechanism of resistance:

Post-transcriptional gene silencing (PTGS) has been the subject of intense investigation in recent years and has also been described as an effective means of resistance to plant viruses (Angell and Baulcombe 1997; Jan, Pang et al. 1999; Savenkov and Valkonen 2001; Lacomme, Hrubikova et al. 2003; Lu, Martin-Hernandez et al. 2003; Baulcombe 2004; Chang, Chen et al. 2005). A number of analyses were performed on C5 plum to further elucidate the mechanism of resistance in C5 plum including: RNA and protein expression; DNA sequencing; nuclear run-on analysis; analysis of transgene methylation; and analysis of the presence of short interfering RNA (siRNA).

Results of the nuclear run-on analysis showed that both C4 and C5 clones had similar levels of PPV-CP RNA transcript (Figure 8, page 32 of petition). This suggests that the low levels of mRNA and non-detectable levels of PPV coat protein found in C5 plum, as described earlier, resulted from post-transcriptional gene silencing.

Another characteristic of PTGS is evidence of transgene methylation (Gonzalez-Zulueta 1995; Elbashir 2001; Turfarelli 2003). Results of restriction digest and Southern blot analysis suggest that the PPV-CP gene sequence in C5 plum is methylated. This determination is based upon larger than expected fragments of *Sau*3A digest probed with a PPV-CP probe. Based upon the results for C3 and C5 plum samples, there appears to be specific methylation of the PPV-CP insert in the C5 plum (Figure 9 and Figure 10, pages 33 & 34 of petition).

Finally, production of siRNA is considered to be diagnostic of PTGS (Angell and Baulcombe 1997; Jan, Pang et al. 1999; Savenkov and Valkonen 2001; Lacomme, Hrubikova et al. 2003; Lu, Martin-Hernandez et al. 2003; Baulcombe 2004; Chang, Chen et al. 2005). Total RNA from C3-2 and C5 was used in northern blot analysis to determine the presence of siRNAs. Samples of inoculated and non-inoculated C5 plum showed the presence of small RNAs of approximately 22 and 25-26 nt (Figure 11, page 35 of petition). These results indicate not only the presence of siRNA in C5 plum, but also that inoculation is not required to induce production of these siRNAs. No siRNAs were detected in either the C3-2 or the non-transgenic plum (Figure 11, page 35of petition) as expected.

Seeds from C5 progeny fruit that resulted from open-pollination experiments conducted at the USDA-ARS research facility in Kearneysville, WV were collected and analyzed. Results of these analyses showed that at one month post-germination, the PPV-CP gene in leaves of seedlings was specifically methylated and produced a similar pattern to the C5 parent (Figure 22, page 56 of petition). In addition, siRNA was detected in ungerminated through four-week post-germination embryo samples. (Figure 25, page57 of petition).

The cumulative RNA analysis data presented, in addition to data collected over multiple years of field trials support the conclusion that the mechanism of resistance for the C5 plum is PTGS. The presence of siRNAs and the lack of detectable protein production are consistent with published literature on gene silencing and the mechanism described for other virus resistant plants.

5. Stability and resistance of C5 plum to PPV

Field trials were performed under appropriate European permits in Poland, Spain and Romania beginning in 1996-1997. The experimental design is described in Section X of the petition and the results of this work are thoroughly described in published literature (Ravelonandro, Monsion et al. 1992; Malinowski, Zawadzka et al. 1998; Hily, Scorza et al. 2004). Briefly, results from the field trials in Poland, conducted with plum lines C2-C6 and a non-transformed control plum, show that the C5 plum was highly resistant to PPV via aphid inoculation, and tolerant to chip bud inoculation with PPV. Despite signs of mild symptoms in chip bud inoculated C5 plum beginning in the second year of the field trial, by year seven of the trial, none of the C5 trees showed symptoms of PPV infection. In contrast, all trees from the other transformed lines (C2-C4 & C6), as well as the non-transformed plum, were infected by year seven. Infection in these other lines started in year one of the trial and increased yearly through year four where there was 95% infection, and finally at year seven when there was 100% infection. Visual symptoms of PPV infection or non-infection were confirmed by use of ELISA, reverse transcription polymerase chain reaction (RT-PCR) and immunocapture RT-PCR (IC-RT-PCR). The IC-RT-PCR test conducted in 2000 revealed the presence of PPV in some leaves of chip bud inoculated C5 trees, but very few if any symptoms. Figures 13 (A) and (B) of the petition (pages 38 & 39) provide details of the plot design and results of the PPV infection analysis.

Further analysis was performed on samples collected from the Poland field trials which compared transgene RNA produced by C3 and C5 plum. Consistent with earlier results, C5 plum produced very small amounts of detectable transgene RNA compared to C3 plum, providing confirmation for the stability of PTGS in C5 plum field trials (Figure 15, page 42 of petition).

Results from both the Spain and Romania field trials corroborated the data obtained in Poland. In both of these trials, both PPV inoculum and aphid vectors were present. Despite adequate virus pressure from two PPV serotypes, and from aphid vectors as evidenced from nearly 100% infection of non-C5 plum trees, none of the C5 trees were infected by PPV (see Figure 17, page 44 of petition). In Spain, the C4 plum showed good

initial resistance against aphid-vectored infection, but once the protection broke down, virus was able to spread throughout the C4 tree.

Data provided and reviewed by APHIS demonstrate stable integration and inheritance of the PPV-CP gene and its associated regulatory sequences over several years of field trials conducted in the U.S. and Europe. Analyses of inheritance showed the expected Mendelian segregation as a single gene dominant trait and stability of the trait through subsequent generations in the breeding program (Table 6, page 55 of petition).

6. Gene Flow from Transgenic Plum

In experiments conducted at the USDA-ARS research station in Kearneysville, WV plum trees that were transformed to express the coat protein of papaya ringspot virus (PRV) were hand-pollinated with pollen collected from C5 plum (Scorza 1995). Fruits that developed from the cross pollination were collected and seeds were removed for further analysis. Figure 18 (page 49 of petition) shows the results of PCR analysis of seedlings produced from these seeds. Of the five seedlings produced from hand-pollination, three contained both the PPV and PRV-CP genes and two contained only the PPV-CP gene. ELISA tests were negative for each plant containing the PPV-CP gene showed that these plants were able to resist PPV infection (Table 5, page 50 of petition).

In greenhouse experiments conducted in France, commercial French *P. domestica* cultivars ('Prunier d'Ente 303' & 'Quetsche 2906') were hand-pollinated with pollen from C5 plum and tested for inheritance of the PPV-CP transgene. Again, resulting fruits were collected and seeds removed for planting and analysis. GUS assays were performed on leaves of putative hybrid seedlings and some seedlings were selected for analysis of resistance to PPV. Positive GUS assays were obtained for 40% and 49% of the 'Quetsche 2906' x C5 and 'Prunier d'Ente 303' x C5 hybrids respectively and these results were confirmed by PCR (Table 6, page 55 of petition). Transgenic hybrids were resistant to infection by PPV. Additional experiments conducted in France and described in Ravelonandro et al. (Ravelonandro 2001b) provided similar results (Table 6, page 55 of petition).

Finally, the open-pollination experiments described previously also provided evidence of transgene inheritance. Fruits were collected from these open-pollinated C5 trees and seeds were removed and analyzed as previously described. Results of a GUS analysis and Southern blot analysis (Figure 20, page 52 of petition) are consistent with stable inheritance of the PPV-CP transgene as a single locus.

Pollen flow experiments were performed with the C5 plum at the USDA-ARS Kearneysville research facility. Very low levels of pollen flow were seen from transgenic to non-transgenic *P. domestica* trees both within a transgenic trial block and between a transgenic block and a non-transgenic block (Figure 26, page 61 of petition). Pollen flow between the transgenic and non-transgenic plum occurred at a distance of 520 m at a rate of 0.067% (2 out of 2,950 seeds) over a six year period.