

December 27, 2004

MEMORANDUM

SUBJECT: Transmittal of Minutes of the FIFRA Scientific Advisory Panel Meeting Held October 13-15, 2004: Issues Associated With Deployment Of A Type Of Plant-Incorporated Protectant (PIP), Specifically Those Based On Plant Viral Coat Proteins (PVCP-PIPs)

TO: Joseph J. Merenda, Director
Office of Office of Science Coordination and Policy

FROM: Paul I. Lewis, Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

THRU: Larry C. Dorsey, Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Please find attached the minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, Virginia from October 13-15, 2004. These meeting minutes address a set of scientific issues being considered by the U.S. Environmental Protection Agency regarding deployment of a type of plant-incorporated protectant (PIP), specifically those based on plant viral coat proteins (PVCP-PIPs).

Attachment

cc:

Susan Hazen
Margaret Schneider
James Jones
Anne Lindsay
Janet Andersen
Debbie Edwards
Steven Bradbury
William Diamond
Arnold Layne
Tina Levine
Lois Rossi
Frank Sanders
George Herndon
William Jordan
Douglas Parsons
Karen Chu
Dayton Eckerson
Enesta Jones
Vanessa Vu (SAB)

FIFRA SAP Members

Stephen M. Roberts, Ph.D.
Steven Heeringa, Ph.D.
Gary Isom, Ph.D.
Kenneth Portier, Ph.D.

FQPA Science Review Board Members

Richard F. Allison, Ph.D.
Ian Cooper, Ph.D.
Jozef J. Bujarski, Ph.D.
Bryce W. Falk, Ph.D.
Steven Gendel, Ph.D.
John Hammond, Ph.D.
Ulrich Melcher, Ph.D.
Peter Nagy, Ph. D.
John Sherwood, Ph.D.
John Stark, Ph.D.
Charles Neal Stewart, Ph.D.
Mark Tepfer, Ph.D.
Milton Zaitlin, Ph.D.

SAP Report No. 2004-09

MEETING MINUTES

**FIFRA Scientific Advisory Panel Meeting,
October 13-15, 2004 held at the Holiday Inn-National
Airport, Arlington, Virginia**

*A Set of Scientific Issues Being Considered by the
Environmental Protection Agency Regarding:*

**ISSUES ASSOCIATED WITH DEPLOYMENT OF A TYPE OF
PLANT-INCORPORATED PROTECTANT (PIP),
SPECIFICALLY THOSE BASED ON PLANT VIRAL COAT
PROTEINS (PVCP-PIPS)**

NOTICE

These meeting minutes have been written as part of the activities of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP). This report has not been reviewed for approval by the United States Environmental Protection Agency (Agency) and, hence, the contents of this report do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the Federal government, nor does mention of trade names or commercial products constitute a recommendation for use.

The FIFRA SAP was established under the provisions of FIFRA, as amended by the Food Quality Protection Act (FQPA) of 1996, to provide advice, information, and recommendations to the Agency Administrator on pesticides and pesticide-related issues regarding the impact of regulatory actions on health and the environment. The Panel serves as the primary scientific peer review mechanism of the EPA, Office of Pesticide Programs (OPP) and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. Food Quality Protection Act Science Review Board members serve the FIFRA SAP on an ad hoc basis to assist in reviews conducted by the FIFRA SAP. Further information about FIFRA SAP reports and activities can be obtained from its website at <http://www.epa.gov/scipoly/sap/> or the OPP Docket at (703) 305-5805. Interested persons are invited to contact Paul Lewis, Designated Federal Official, via e-mail at lewis.paul@epa.gov.

In preparing these meeting minutes, the Panel carefully considered all information provided and presented by the Agency presenters, as well as information presented by public commenters. This document addresses the information provided and presented within the structure of the charge by the Agency.

TABLE OF CONTENTS

	Page
Participants.....	7
Public Commenters.....	8
Introduction.....	9
Introductory Comments.....	9
Summary of Panel Discussion and Recommendations.....	10
Panel Deliberations and Response to the Charge.....	13
References.....	51

SAP Report No. 2004-09

**MEETING MINUTES:
FIFRA Scientific Advisory Panel Meeting,
October 13-15, 2004, held at the Holiday Inn- National
Airport, Arlington, Virginia**

*A Set of Scientific Issues Being Considered by the
Environmental Protection Agency Regarding:*

**ISSUES ASSOCIATED WITH DEPLOYMENT OF A TYPE
OF PLANT-INCORPORATED PROTECTANT (PIP),
SPECIFICALLY THOSE BASED ON PLANT VIRAL COAT
PROTEINS (PVCP-PIPS)**

Paul I. Lewis, Ph.D.
Designated Federal Official
FIFRA Scientific Advisory Panel
Date: December 27, 2004

Steven Roberts, Ph.D.
FIFRA SAP Session Chair
FIFRA Scientific Advisory Panel
Date: December 27, 2004

**Federal Insecticide, Fungicide, and Rodenticide Act
Scientific Advisory Panel Meeting
October 13-15, 2004**

**Issues Associated With Deployment Of A Type Of Plant-Incorporated Protectant (PIP),
Specifically Those Based On Plant Viral Coat Proteins (PVCP-PIP)**

PARTICIPANTS

FIFRA SAP Session Chair

Stephen M. Roberts, Ph.D., Professor and Program Director, Center for Environmental and Human Toxicology, University of Florida, Gainesville, FL

Designated Federal Official

Paul Lewis, Ph.D. FIFRA Scientific Advisory Panel, Office of Science Coordination and Policy, U.S. Environmental Protection Agency, Washington, D.C.

FIFRA SAP Members

Steven G. Heeringa, Ph.D., Research Scientist & Director for Statistical Design
Institute for Social Research, University of Michigan, Ann Arbor, MI

Gary Isom, Ph.D., Professor of Toxicology, School of Pharmacy & Pharmacal Sciences
Purdue University, West Lafayette, IN

Kenneth Portier, Ph.D., Associate Professor, Statistics, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL

FOPA Science Review Board Members

Richard F. Allison, Ph.D., Associate Professor of Plant Biology, Department of Plant Biology, Michigan State University, East Lansing, MI

Ian Cooper, Ph.D., Senior Research Scientist, Natural Environment Research Council, Center for Ecology and Hydrology, Oxford, United Kingdom

Jozef J. Bujarski, Ph.D., Professor of Biological Sciences, Department of Biological Sciences, Northern Illinois University, DeKalb, IL

Bryce W. Falk, Ph.D., Professor, Department of Plant Pathology, University of California at Davis, Davis, CA

Steven Gendel, Ph.D., Branch Chief, National Center for Food Safety and Technology, Food and Drug Administration, Summit-Argo, IL

John Hammond, Ph.D., Research Leader, Floral and Nursery Plants Research Unit, USDA-
Page 7 of 54

ARS, U.S. National Arboretum, Beltsville, MD

Ulrich Melcher, Ph.D., Robert A. Sirny Professor of Agricultural Biotechnology, Department of Biology and Molecular Biology, Oklahoma State University, Stillwater, OK

Peter Nagy, Ph.D., Associate Professor, Plant Pathology Department, University of Kentucky, Lexington, KY

John Sherwood, Ph.D., Professor and Head, Department of Plant Pathology, University of Georgia, Athens, GA

John Stark, Ph.D., Professor, Department of Entomology, Washington State University Puyallup, WA

Charles Neal Stewart, Ph.D., Professor and Racheff Chair of Excellence in Plant Molecular Genetics, Plant Sciences, University of Tennessee, Knoxville, TN

Mark Tepfer, Ph.D., Senior Research Scientist, INRA-Versailles, Laboratoire de Biologie Cellulaire, Versailles Cedex, France

Milton Zaitlin, Ph.D., Professor Emeritus, Department of Plant Pathology, Cornell University, Ithaca, NY

PUBLIC COMMENTERS

Oral statements were presented as follows:

On behalf of United States Department of Agriculture, Animal Plant Health and Inspection Service:

John Turner, Ph.D.

On behalf of the American Phytopathological Society

Susan Tolin, Ph.D., Virginia Polytechnic Institute and State University

Written statements were provided by or on behalf of the following group:

American Phytopathological Society

INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP) has completed its review of a set of scientific issues being considered by the Agency pertaining to its review of the issues associated with deployment of a type of Plant-Incorporated Protectant (PIP), specifically those based on plant viral coat proteins (PVCP-PIPs). Advance notice of the meeting was published in the *Federal Register* on September 10, 2004. The review was conducted in an open Panel meeting held in Arlington, Virginia, from October 13-15, 2004. The meeting was chaired by Stephen Roberts, Ph.D. Paul Lewis, Ph.D. served as the Designated Federal Official. Mr. Joseph Merenda, Jr. (Director, Office of Science Coordination and Policy, EPA) welcomed the Panel to the meeting. Mr. Dennis Szuhay (Chief, Microbial Pesticides Branch, Biopesticides and Pollution Prevention Division, Office of Pesticide Programs, EPA) offered opening remarks at the meeting. Elizabeth Milewski, Ph.D. (Office of Science Coordination and Policy, EPA) provided context of PVCP-PIPs. Anne Fairbrother, DVM, Ph.D. (National Health and Environmental Effects Research Laboratory, ORD, EPA) discussed gene flow in viral coat protein transgenic plants and Melissa Kramer, Ph.D. (Office of Science Coordination and Policy, EPA) summarized viral interaction in viral coat protein transgenic plants. Elizabeth Milewski, Ph.D. (Office of Science Coordination and Policy, EPA) completed the Agency's presentation by discussing other technical considerations and a review of the charge to the Panel.

INTRODUCTORY COMMENTS

The use of terminology in the regulatory and scientific arenas are not always the same. To facilitate the readers' understanding of the context of the questions developed by the Agency and the responses of the FIFRA SAP, the following terms were defined:

From the Agency's Background Document "Plant Incorporated Protectants Based on Plant Viral Coat Proteins" (page 14 of 14), the Agency defined the following terms:

resistant: when referring to PVCP-PIPs only, means the plant is not infected by or is a non-host of the virus concerned.

tolerant: when referring to PVCP-PIPs only, means that the plant is able to sustain the effects of a virus infection with negligible or mild symptom expression and negligible or mild effects on fitness or growth despite the presence of the virus within the host (limited to phenotypic expression as considered by the Panel).

In meeting the charge to the FIFRA SAP, it is necessary to make comparisons between plants with PVCP-PIPs and either wild or cultivated plants without PVCP-PIPs in regards to susceptibility to virus infection, the scope or limit of infection, virus replication and distribution, and the final phenotypic expression of the interaction of the virus and the plant, i.e. disease. As these minutes are to reflect the conclusions of the Panel, the following terms are used in the responses of the Panel. These terms, when used as defined by the FIFRA SAP, will appear in italics with quotations as below. Readers of these minutes are urged to seek other refereed sources, specifically Cooper and Jones (1983) and Hull (2002) that were not available to the Panel at the time of the meeting for a more thorough discussion of terms related to the reaction of plants to virus infection.

“*immunity*”: virus does not replicate in host (equivalent to the Agency’s definition of resistance for PVCP-PIPs)

“*subliminal infection*”: virus can replicate in the host, but spread is limited, no readily observable altered phenotype compared to uninfected plant, i.e., no disease

“*resistant*”: virus generally limited in its replication and or spread in plant, but primary expression of “*resistance*” in cultivated plants is lack of symptoms or appreciably reduced symptoms compared to an infected susceptible genotype, and in non-cultivated plants is a lack or reduction of symptoms typically associated with the phenotypic expression of disease in cultivated plants (i.e., mosaic, stunting, necrosis, foliar deformity). This is equivalent to the EPA definition of tolerance for PVCP-PIPs.

“*tolerance*”: when a host contains readily detectable amounts of virus but lacks significant disease symptoms and growth/performance is not obviously affected (both phenotypic and viral titer expression as considered by the Panel).

SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

Gene Flow

The effect of virus infection is very much dependent on the specific virus - host interaction whose outcome in regard to disease can be quite variable. Our knowledge about the effect of virus infection on non-crop plants is quite limited. What has been observed is that the impact of virus infection on non-crop plants is as variable as with cultivated plants. Virus infection, regardless of the plant being a crop or non-crop plant, may negatively impact some aspect of plant development and reproductive capacity. The Panel was not aware of any study that has purposefully freed a weed species of a known virus and determined its competitive ability or looked at the population dynamics of virus infected versus uninfected plants in multiple species ecosystems. Based on knowledge obtained from observation of cultivated crops in the agroecosystem, the majority of the Panel concluded that it would be unlikely that a plant population freed from viral pressure would give a plant species a competitive advantage.

The Agency list of plants with no wild relatives in the US - tomato, potato, corn, and soybean, is adequate insofar that it lists only crops of large acreage in the US. The Panel concluded that a number of important crops are not expected to form viable hybrids with wild relatives in the US and US territories, primarily fruit and vegetable species.

Even though introgression happens infrequently, and the consequences of introgression can be controlled overridingly by genes linked with the transgene, there is a slight chance that a boost in tolerance to a wild plant could be ecologically significant.

Overall Panel members were of the opinion that PVCP-PIPs had no inherent capacity to harm the environment. The Panel accepted that processes aiming at diminished gene exchange

are desirable (even necessary) but are not sufficient and are not themselves hazard free. When setting limits for the sampling of wild relatives for the occurrence of virus tolerance / resistance, the Panel suggested biogeographic regions such as the USDA “hardiness zones”. The Panel was aware of imperfections in the conditions suggested by the Agency but no other conditions were identified as better for the purpose.

In general, methods utilizing sexual crosses are most useful for beginning predictions of gene flow in the field, whereas many lab-intensive methods are not helpful to this end.

Most members of the Panel were persuaded of the possibility that gene escape could go unrecognized for a significant time period thus enabling transgene spread within the natural population to a point where eradication could not be achieved. The Panel agreed that experimental evidence informing this aspect of hazard assessment is critically needed and it is now timely to investigate particular crop-relative combinations to illuminate the questions of impact consequent upon gene flow from wild to cultivated relatives.

“*Resistance*” and/or “*tolerance*” were accepted by the Panel to be common among relatives of crop plants.

Mechanical inoculation of plants with viruses can be useful as a first pass to determine if a wild plant or crop variety is tolerant to the virus.

Environmental factors, notably the temperature under which diseased plants are grown, have an influence on “*tolerance*” and/or “*resistance*.” In some instances “*resistance*” may be overcome by an increase in temperature. “*Resistance*” in plants obtained by selection or by plant breeding is subject to ‘breakdown’ by viral variants (generated within the plant’s viral population) that can overcome the “*resistance*” and increase the disease. To date, there have not been any instances of ‘breakdowns’ of PVCP-PIPs, suggesting it is a durable resistance strategy.

Viral Interactions

Heterologous encapsidation, synergy and recombination are not novel viral interactions as such, since they are phenomena that have been shown to occur in plants infected by more than one virus. The question that was explored by the Panel is whether these interactions could have novel effects in PVCP-PIPs, compared to their effects in non-transgenic plants. There was good agreement within the Panel that neither heterologous encapsidation nor synergy should be considered to be of serious concern. Although there is no *a priori* evidence that recombination in plants expressing a PVCP-PIP would lead to creation of recombinant genomes that would not also occur in non-transgenic plants, concern was expressed about certain limited situations.

Although unique characteristics of some challenging viruses and viral transgenes may influence recombination frequencies in PVCP-PIPs, the Panel felt that in general recombination was more likely to occur in transgenic plants than in non-bioengineered plants. Whether or not it might lead to viable viruses is unknown. Heterologous encapsidation has been demonstrated in both non-bioengineered plants and transgenic plants expressing functional coat proteins.

Heterologous encapsidation should be anticipated for compatible combinations of transgenic coat protein and challenging virus. However, no increase in heterologous recombination or unique combinations of coat protein and viral RNA should be anticipated in PVCP-PIP plants.

The Panel agreed that deleting the 3' UTR may reduce the frequency of recombination in certain viral systems but may not be effective in all viral systems. Whenever possible, other methods should be considered to decrease the recombination frequency, and a combination of several methods was recommended. However, none of these methods is sufficiently effective to ensure that recombinants could not occur. Thus, the surveillance of recombination events might be necessary.

The Panel agreed that universally applicable methods were not available for inhibiting heterologous encapsidation or insect vector transmission, but that methods were generally available on a case-by-case basis if either of the above-mentioned events posed a real risk.

The Panel agreed that it would be technically feasible, though often challenging, to measure rates of recombination, heterologous encapsidation and vector transmission to determine whether modification of the PVCP gene reduces these processes. In assays for all three processes, Panel members felt that one of the most challenging tasks was the design, execution and validation of appropriate controls for the assays.

The Agency's four strategies for reducing risk of novel virus interactions are of modest value. For these four strategies, there were two types of objections. One was based on the relatively ineffective nature of these measures. If one really wishes to eliminate novel interactions, then resistance based on post-transcriptional gene silencing is an obvious solution. The second type of objection, raised by several Panel members, was that the potential novel interactions are not of serious concern, and thus did not merit that such measures be taken.

Other Questions

The Panel agreed that (because of the human history of consuming virus infected food), unaltered PVCPs do not present new dietary exposures. There was no clear consensus on how much change would be necessary to invalidate this assumption, although there was general agreement that the appropriate comparison is to the range of natural variation in the virus population.

The Panel agreed that virus-protected crops should not be as attractive to aphids as diseased crops and thus may reduce the numbers and types of organisms that spend at least part of their life cycles in specific agroecosystems. However, because many agroecosystems are transient in nature, the impact of any changes in numbers of aphids in PVCP-PIP plants should be negligible over larger scales such as the landscape. Additionally, the Panel was not aware of any risk posed by PVCP-PIPs crops to wildlife.

Modifications that fall within the natural variability of the PVCP were considered to be of little concern by the Panel. More important changes would be more likely to reduce the

effectiveness of the PCVP than to cause new interactions to occur. Some concern regarding insertions into the PVCP gene was expressed.

The Panel could not envisage additional requirements related to PVCP-PIP identity and composition. Finally, no potential risks beyond the previously discussed issues of recombination, heterologous encapsidation, and synergy were identified.

PANEL DELIBERATIONS AND RESPONSE TO THE CHARGE

The specific issues to be addressed by the Panel are keyed to the Agency's background documents, references and Agency's charge questions.

Gene Flow

Concerns about the transfer of virus resistance to wild or weedy relatives include the assumption that such resistance might confer a selective advantage to a wild or weedy relative that could increase its competitive ability and potential to become weedy or invasive. The Agency would like the Panel to consider the evidence supporting this assumption.

1a. What scientific evidence supports or refutes the idea that plant viruses have significant effects on reproduction, survival, and growth of plant populations in natural settings?

Panel Response

The effect of virus infection is likely dependent on the specific virus - host interaction whose outcome in regard to disease can be quite variable. Our knowledge about the effect of virus infection on non-crop plants is quite limited. What has been observed is that the impact of virus infection on non-crop plants is as variable as with cultivated plants. Virus infection, regardless of the plant being a crop or non-crop plant, may negatively impact some aspect of plant development and reproductive capacity.

Agroecosystems are not “natural settings.” Production areas, even when viewed beyond the borders of a field or fields, have long been disturbed. The Panel was not familiar with any literature that provides an extensive inventory of plant viruses in an undisturbed ecosystem. Thus, for this comment a “natural setting” is defined as areas adjacent to production areas. As viruses are obligate parasites, it would be an evolutionary dead end if viruses impacted their hosts or vectors too significantly. Previously not described viruses continue to “emerge” in crops, likely from outside the crop, indicating viruses likely do not significantly impact non-crop plants. Our primary knowledge about the effects of viruses on plants comes from cultivated plants, and the obvious goal with cultivated plants is to lessen the impact of virus infection on plant growth and subsequent yield of the plant part of commercial interest. As stated previously, the effect of virus infection is dependent on the specific virus - host interaction whose outcome in regard to disease can be quite variable. Macroscopic systems can include reduction in growth, reduction in vigor, reduction in quality or infection may be masked (Hull 2002). While we do

know something about the impact of virus on cultivated plants, our knowledge about the effect of virus infection on non-crop plants is quite limited. What has been observed is that the impact of virus infection on non-crop plants is as variable as with cultivated plants.

Most virus epidemics result from the viruses and/or vectors coming from non-crop plants adjacent to production areas. If the hosts in these natural settings are too adversely affected by the virus or vector, the epidemic cycle would be broken as the plant reservoir for virus and vector would no longer be present. As summarized by Duffus (1971), “From the standpoint of control of virus diseases, there is perhaps no phase of virology more important than epidemiology. The role of weeds in the occurrence and spread of plant virus diseases is an integral part of the ecological aspect of virus transmission.” Hence the primary question on the impact of viruses on “natural settings” is what is the impact of viruses on weeds? The literature is filled with reports of different viruses on different plant hosts, either found in natural infections or purposefully inoculated, as plant host range has long been a method to differentiate viruses and virus strains. Many virus infections in weeds do not produce visible symptoms, and some have been reported to even protect plants from herbivores (Gibbs 1980) or other plant pathogens. Additionally, examples were given of *Plantago sp.* (Hammond 1982), *Nicotiana glauca*, and spinach with infections by up to 4, 7 and 9 different viruses, respectively, without apparent visible adverse effects. However, a significant body of literature on the effect of viruses on weed species is lacking. Friess and Maillet (1997) found that *Cucumber mosaic cucumovirus* (CMV) infected chickweed plants (*Stellaria media*) grown in a monoculture had similar vegetative production to a monoculture of control healthy plants. However, when healthy and infected plants were grown together, as the density of healthy plants grown with infected plants increased, infected plants were not as vegetatively productive or as reproductive. Work from this lab on nitrogen partitioning in CMV infected versus healthy weeds found no difference in virus infected and healthy chickweed plants, but nitrogen partitioning to shoots and roots was different in CMV infected and healthy purslane (*Portulaca oleacea*) (Navas *et al.* 1998). Remold (2002) examined the incidence of *Barley yellow dwarf luteovirus* (BYDV) in three grass hosts, soft brome grass (*Bromus hordeaceus*), green foxtail (*Setaria viridis*), and yellow foxtail (*Setaria lutenscens*). Using panicle length as a measure of fitness, soft brome grass was not affected by virus infection, fitness of green foxtail was about half of uninfected plants and infected yellow foxtail had about 25% greater fitness than uninfected plants. Maskell *et al.* (1999) found that wild cabbage (*Brassica oleracea*) inoculated with either *Turnip mosaic potyvirus* (TuMV) or *Turnip yellow mosaic tymovirus* had significantly reduced survival, growth and reproduction. In a recent three year study of CMV in Central Spain, Sacristán *et al.* (2004) found that the incidence of CMV in weeds fluctuated in various habitats (fallow fields, edges and wastelands) through the growing season with a maximum incidence of 20-30% in summer and autumn. The greater amount of biomass and soil cover was correlated with the greater incidence of CMV. Thus, there is quite a bit of variation in the inferred impact of viruses on plant growth.

Virus infection, regardless of the plant being a crop or non-crop plant, may negatively impact some aspect of plant development and reproductive capacity. For weed species our knowledge is limited to the fitness (i.e. any selective advantage or disadvantage conferred to the plant) of an individual or individuals in a population, as there appears to be a lack of published

information on longer term studies of the effects of recurring virus infection in different weed populations. Genetic non-uniformity of wild plant populations complicates prediction and measurement of impacts on wild plant survival, but we have some data from the annual species, *Brassica rapa*. Within the European Union funded VRTP-IMPACT program, analyses have been made on two populations on one annual species (*B. rapa*), but the data are not yet robust enough to be extrapolated to other sites/genotypes of the species in question. Because of the effort (and labor cost) there is no immediate prospect of similar measurements being made with any perennial species (including *B. oleracea*). The field observations to identify the key stages in the *B. rapa* life cycle were initiated against a background of surveys of virus prevalence in natural settings and extensive greenhouse challenge data in the context of TuMV. These observations showed that while virus isolates may differ in their effect on the plant, generally a virus lessens the vigor of the seedlings and plausibly lessens their performance in competition with neighbors. Key pieces of information are needed at a community level on the effect of virus infection on fecundity, adaptation, survivability and competition of non-crop plants.

1b. Is there scientific evidence that plant populations freed from viral pressure could have increased competitive ability leading to changes in plant population dynamics?

Panel Response

The Panel was not aware of any study that has purposefully freed a weed species of a known virus and determined its competitive ability or looked at the population dynamics of virus infected versus uninfected plants in multiple species ecosystems. Based on knowledge obtained from observation of cultivated crops in the agroecosystem, the majority of the Panel concluded that it would be unlikely that a plant population freed from viral pressure would give a plant species a competitive advantage.

Current production practices also provide an indirect test of competitiveness of “plant populations freed from viral pressure.” When a virus is controlled in a crop plant population by “resistance,” that is manifest by low virus titer, the inoculum load of virus that can move to alternate, wild hosts (weeds) is impacted. Thus, controlling a virus in a crop reduces virus pressure to alternate host plants. This has been done for years in conventional agriculture by “resistance” and other virus disease control methods with no evidence of increased weed pressure.

In regards to plant population dynamics, the effect of *Tomato spotted wilt tospovirus* on peanut plant populations is a good demonstration of the effect a virus can have on population dynamics. Fields of peanut plants that have no or little virus are more competitive in that vines “lap” earlier blocking the sun between rows thus reducing weed pressure. The significant study of Jones and Nicholas (1998) in self-regenerating pasture in Australia over a four year period provides a good look at the introduction of virus in a multiple species complex environment. They sowed seeds of burr medic (*Medicago polymorpha*) that were either free or infected with *Alfalfa mosaic alfamovirus* (AMV) in mixed species pastures and followed the effect on proportion of species over time. Generally, less desirable species became established as the virus became established, but the effect varied with medic cultivar. Difficulty in determining the

effect was compounded with the extent of aphid abundance (that transmits AMV) which was variable. The Panel was not aware of any reports on the impact of virus infection on plant competition in non-agricultural settings.

2. Please comment on the validity of the Agency list of crops that have no wild or weedy relatives in the United States with which they can produce viable hybrids in nature (i.e., tomato, potato, soybean, and corn).

Panel Response

The Agency's list of plants with no wild relatives in the US - tomato, potato, corn, and soybean, is adequate insofar that it lists only crops of large acreage in the US. One panelist mentioned that tomato has a number of properties that are significant in the context of potential invasiveness; passage of viable seed through vertebrate (notably rodent) digestive systems is important but so too is the potential of the genus (*Lycopersicum*) to be a perennial rather than annual.

3. Please identify other crops that have no wild or weedy relatives in the United States with which they can produce viable hybrids in nature, e.g., papaya, peanut, and/or chickpea.

Panel Response

The Panel concluded that a number of important crops, primarily fruit and vegetable species, are not expected to form viable hybrids with wild relatives in the US and US territories (as defined by the Agency). The formation of viable hybrids might occur when crop and wild relative co-occur and can cross. The Panel drew a distinction in lifetime probabilities of hybridization participation: perennial plants have a better chance of parenting hybrids during their lifetimes compared with annuals. Where annual crops are grown, they can change year to year (deployed in time) from crop-to-crop whereas perennials can be long-lived. Wild relatives of crops could be volunteer weeds or naturalized plants of the same species, or of a closely related, truly wild species. Crops can also be naturalized and then considered wild or feral in some instances. Volunteers might appear but are often transient in nature. The question by the Agency pertains to wild relatives *per se* and the potential to form viable hybrids with crops, realizing that most hybrids are evolutionary dead ends. The Panel produced a conservative list of crops with no wild relatives in the US, protectorates and territories (Table 1). The candidates were taken from the USDA National Agricultural Statistical Service (NASS, www.usda.gov/nass) listings of crops grown in specific states, protectorates or territories, and species were eliminated if literature suggested crop x wild hybridization could occur. The NASS does not name certain commodities including the cut flowers, nursery crops and bedding plants. There is a need to assess which of these crops might be exempt from crop x wild hybridization in the US, but such an assessment was outside the purview of the FIFRA SAP. A second list of plants that have been transformed is included as a starting point (Table 2).

Table 1. Crops that will not form viable hybrids with wild relatives in the US and US territories.

almond (*Prunus communis*)
 apricot (*Prunus armeniaca*)
 asparagus (*Asparagus officinale*)#
 avocado (*Persea americana*)
 banana (*Musa acuminata*)
 barley (*Hordeum vulgare*)
 bean (*Phaseolus vulgaris*)
 black-eyed pea (cowpea) (*Vigna unguiculata*)
 cacao (*Theobroma cacao*)
 celery (*Apium graveolens*)#
 chickpea (*Cicer arietinum*)
 citrus (*Citrus* spp.)
 coffee (*Coffea arabica*)
 cucumber (*Cucumis sativus*)
 eggplant (*Solanum melongena*)
 guava (*Psidium guajava*)
 kiwi (*Actinidia* spp.)
 mango (*Mangifera indica*)
 nectarine (*Prunus persica*)
 okra (*Abelmoschus esculentus*)
 olive (*Olea europaea*)
 papaya (*Carica papaya*)
 parsley (*Petroselinum crispum*)
 pea (*Pisum sativum*)
 peach (*Prunus persica*)
 peanut (*Arachis hypogaea*)
 pineapple (*Ananas comosus*)
 pistachio (*Pistacia vera*)
 plum (*Prunus domestica*)
 spinach (*Spinacia oleracea*)
 starfruit (*Averrhoa carambola*)
 sugar cane (*Saccharum officinarum*)*
 taro (*Colocasia esculenta*)
 tobacco (*Nicotiana tabacum*)+
 watermelon (*Citrullus lanatus*)

Notations:

can escape cultivation—forms occasional volunteer populations

* not applicable in Puerto Rico, Virgin Islands, Guam, Trust Territories of the Pacific Islands, American Samoa

+ does have a few congeners but is a highly selfing plant—no evidence of hybrids in the US

Table 2. Ornamental crops that are not expected to form hybrids with wild relatives in the United

States

Anthurium

Carnation

Chrysanthemum

Geranium (Pelargonium)

Gerbera

Gladiolus

Hyacinth

Orchids (various – Oncidium, Dendrobium, Calanthe, Cymbidium, Phalaenopsis,)

Osteospermum

Petunia

Torenia

Tulip

The Agency Anticipates The Need To Evaluate Data Addressing Whether Transgenic Plant Species Are Capable Of Genetic Exchange With Wild Or Weedy Plant Relatives. In General, EPA Is Focused On The Potential For Genetic Exchange That Can Occur In The Field. However, Evaluations Of The Potential For Genetic Exchange Are Likely To Include Laboratory Studies That Are Not Necessarily An Accurate Indicator Of Plants' Ability To Exchange Genetic Material Outside The Lab.

4. What Laboratory Techniques Used To Achieve Genetic Exchange Between Species (E.G., Embryo Rescue, Use Of Intermediate Bridging Crosses, Protoplast Fusion) Are Not Indicative Of Possible Genetic Exchange Between These Species In The Field? Conversely, What Techniques, If Any, Used In Laboratory Or Greenhouse Experiments Provide The Most Reliable Indication Of Ability To Hybridize In The Field?

Panel Response

In general, methods utilizing sexual crosses are most useful for beginning predictions of gene flow in the field, whereas many lab-intensive methods are not helpful to this end.

Lab-intensive methods to combine germplasm, such as embryo rescue, protoplast fusions, emasculation, chromosome injection, bridge-crossing, application of pollen to manipulated pistils, application of pollen to cleistogamous flowers, and seasonal pollen storage are not predictive of gene flow in the field.

Hand crosses in growth chamber and greenhouses are useful as first approximations of the ability to hybridize and backcross using reciprocal crosses (Halfhill et al. 2001, Zhu et al. 2004 and Halfhill et al. 2004). When using the same *Brassica napus* transgenic events, hybridization frequencies were 3 to 7 times lower in field than in handcrosses, but backcrossing rates were about two orders of magnitude less in the field. Furthermore, hand crosses can demonstrate if species are sexually compatible and if a transgene at a particular locus is potentially transmittable (Stewart et al. 2003). One factor to bear in mind is that there is often

genotype dependence with regards to hybridization ability and frequency. There are techniques in greenhouse experiments to mimic insect-mediated and wind-mediated pollination to refine estimations.

In the field, there are factors that could prevent hybridization and introgression including non-overlapping flowering times, pollen competition, non-selection of the target trait in progeny, linkage disequilibrium, genetic exclusion, and intra- and inter-specific competition within plant communities (Stewart et al. 2003).

5. Given that current bioconfinement techniques are not 100% effective, what would the environmental implications be of extremely low transfer rates of virus-resistance genes over time?

Panel Response

Most members of the Panel were persuaded of the possibility that gene escape could go unrecognized for a significant period thus enabling transgene spread within the natural population to a point where eradication could not be achieved. The Panel agreed that experimental evidence informing this aspect of hazard assessment is critically needed and it is now timely to investigate particular crop-relative combinations to illuminate the questions of impact consequent upon gene flow between cultivated and wild relatives.

When setting the context for the question, the Agency had cited some literature (Haygood et al. 2004) which argues that even very low transmission rates for transgenes to wild populations can eventually result in fixation of the genes. One member of the Panel did not accept the prediction from that mathematical model. Some other members of the Panel were not satisfied with the evidence suggesting that viruses play a role in weed/natural plant survival. It was generally agreed that better bioconfinement methods might emerge in the future but all accepted that, given sexually compatible recipients, we should plan for gene escape.

In this context there was substantial discussion but most Panel members supported the possibility that a slowly increasing impact that may escape recognition early enough to allow eradication of a problem was a realistic outcome in light of the Agency's question. Many uncertainties about the variability of crop to wild relative gene flow were recognized but the biggest gap in knowledge concerns the magnitude of actual resulting harm. If the transgene was linked to agronomic traits including large seed size, high nutrient needs, etc., it was suspected that the genetic linkage drag would be such that no stable introgression would occur. However, the Panel agreed that this benign outcome could not be assumed. For a variety of poorly understood reasons the experience has been that hybrids survive poorly. Nevertheless, complex, and presumably very rare, genetic events do get perpetuated (the genomes of *Brassica* species provide examples of complex evolution involving polyploidy, chromosomal chimeras and duplications; Truco et al., 1996).

Transgenes for virus resistance/tolerance, etc. have been shown (experimentally) to transfer from crops to wild relatives but these observations have not been designed to show

introgression into populations of the wild relatives (or impacts) in the field.

Many wild plants contain virus “*resistance*” genes but the sources of those genes are not known. The Panel was not aware of any concerns that have been raised by farmers or harm that was known to have resulted from flow of virus “*resistance*” genes. However, the Panel agreed that it is now timely to investigate particular crop-relative combinations to illuminate this question of gene flow between wild and cultivated relatives. The Panel agreed that there is no evidence suggesting that any natural genes for virus “*resistance*” / “*tolerance*,” etc. have naturally introgressed into crop cultivars (although plant breeders seeking to sustain crop productivity routinely use the same genes). Genomic tools allowing appropriate monitoring of gene flow from crops to wild relatives have not hitherto been available but are now beginning to be developed. Experience with viruses and wild *Brassica* species in the United Kingdom has revealed such complex interactions involving different viral pathotypes and genetic diversity in the plants that prediction of outcomes must be case specific and cannot be generic. Nevertheless, building on the extensive prior work, *Brassica* systems offer attractive opportunities; specifically as a result of the pathotype-specific turnip mosaic virus (TuMV) “*TuRBO*” “*resistance*” genes (e.g. Walsh et al. 1999) that occur naturally (as plant genes) and have already been characterized and mapped. Individual genes in the *TuRBO* series provide ideal surrogates for PVCP-PIPs and the field use of *Brassica* genotypes containing the natural genes is not regulated.

When hybridization and stable introgression are possible, the Panel recognized that genes from crops may increase in frequency when they confer greater lifetime fitness on the individuals with the gene than those without it. However, the near complete absence of data on whole life fitness characteristics of any wild species was noted. It was agreed that hybrid genotypes will not necessarily become either more persistent or invasive but the Panel generally agreed there could be an effect on biodiversity. Among the possible outcomes are disruption of genetic integrity of local ecotypes, or the hybrid-derived wild species may become more genetically uniform than hitherto. Additionally, the Panel recognized that there might be fixation of the new genotype in scattered sub-populations.

When a wild species was known to be sufficiently sexually compatible for gene flow to occur at any frequency, the assumption should be made that the probability of hybridization over time will be 1.

EPA recognizes that concerns about gene flow to wild or weedy relatives may be ameliorated if the introduced virus-resistance trait would give little or no selective advantage to the recipient plant, as would occur if the plant were already tolerant or resistant to the virus to which resistance is conferred. It is obvious that such resistance does exist in some populations because traditional breeding for resistance relies on finding a source of resistance within related cultivated species, old varieties, or wild species.

6. Please comment on the prevalence of tolerance and/or resistance to viruses in wild relatives of crops.

Panel Response

“Resistance” and/or “tolerance” were accepted by the Panel to be common among relatives of crop plants. Most plants are “resistant” to most pathogens (viruses). “Tolerance” and “resistance” to indigenous pathogens/viruses are each present in wild populations of many plants. The phenotypes for these range from supporting virus replication but having no symptoms (“tolerance”), to limited infection foci (“subliminal infections” in a single or few cells), to no infection (“resistance”). Some of these traits are due to single genes while others are multi-genic traits. Plant breeders have consistently searched for germplasm sources to use in breeding programs in order to mitigate effects of plant viruses in crop plants.

In the book, *Resistance to Viral Diseases of Vegetables: Genetics and Breeding* (1993), examples are given of virus “resistance” sources for cucurbits, lettuce, peppers, tomatoes, peas and beans. In various chapters the different authors document sources of genetic “resistance”, such as the use of landraces, etc. Lettuce (*Lactuca sativa*) “resistance” to *Lettuce mosaic potyvirus* (LMV) comes from related species such as *L. virosa*, *L. saligna* and *L. serriola*. In the last chapter of the book, Future Prospects, Sorenson et al. state “most of the sources of “resistance” (quotes and italics added) that we utilize in breeding programs come from foreign germplasm and we are increasingly relying upon wild relatives of cultivated species for viral “resistance” (quotes and italics added) genes”. Another specific example of virus “resistance” is the *yd2* gene which confers “resistance” to *Barley yellow dwarf luteovirus* (BYDV) in barley. This gene was recovered by conventional plant breeding using a BYDV “resistant” Ethiopian landrace of barley.

Another way to look at virus “resistance” among wild plants is when viruses suddenly appear in new crops, or new crops to a given area. *Cacao swollen shoot badnavirus* in Africa is a frequently cited example of inapparent infection until agricultural plants, cacao (*Theobroma cacao*), were introduced into an area of west Africa. The virus spread from symptomless reservoir plants, which were never identified, and commercial production of cacao was severely affected, resulting in losses of millions of trees. It is suspected that the virus was indigenous in the native plants, causing no detectable effects, but did cause disease when a susceptible population (cacao) was present. Similar examples are available, such as the widespread incidence of symptomless virus infections in *Plantago*, and the glasshouse-determined “resistance” (or “immunity”) to *Turnip yellow mosaic tymovirus* (TYMV) reported by Thurston et al. (2001). TYMV was not detected in *B. nigra* at one site but was common elsewhere. When *B. nigra* seedlings from this site were challenged manually with TYMV, 21/31 of the individual plants were not infected. This frequency of immunity [or using the Agency’s term “resistance”] was not observed when *B. nigra* seedlings from other places were inoculated. Both of the examples above support the view that “resistance” among wild plant species is the norm.

Even if there is not documented “resistance” in wild plant species, it has been argued that viruses become pathogens of wild plants when susceptible crop plants are grown in their vicinity (Duffus 1971). If the viruses move into the susceptible crop plants, this can present a new and significant source of inoculum for subsequent spread into the native plants.

7. Please specify techniques that do or do not provide measures of tolerance and/or resistance that are relevant to field conditions.

Panel Response

Mechanical inoculation of plants with viruses can be useful as a first pass to determine if a wild plant or crop variety is tolerant to the virus. Screening any plants, crop or native plants, only in the greenhouse/field by mechanical means of inoculation, does not necessarily adequately reflect field conditions. In general, mechanical inoculation would increase inoculum load more than is encountered naturally in the field. Thus, susceptibility from mechanical inoculation must be interpreted to reflect just that: susceptibility to mechanical inoculation. Under natural conditions, vector preference is an important characteristic in determining virus incidence. Leaf pubescence affects aphid probing/feeding behavior and thus if aphids are the vector of specific viruses, leaf pubescence can give effective field “*resistance*” even for plants that are virus susceptible. Similarly, if a virus is transmitted to plants by vectors that must feed in phloem tissues for inoculation to be successful, if the vector cannot find the phloem this is effective “*resistance*” (e.g. BYDV and aphid vectors).

That said, there is merit to a variety of mechanical inoculation experiments (growth chamber, greenhouse, and the field as resources permit) for first approximations to gauge “*tolerance*,” “*resistance*,” or susceptibility of any plant to a particular viral strain. There is a chance of observing false positives: plants that do not seem “*resistant*,” when they really might be the result of experimental artifacts. It is preferable to test a number of viral strains and plant genotypes. If a virus has more than one effective vector and if “*resistance*” screens use only one vector [biotypes or ecotype], misleading data can be generated if the plants exhibit “*resistance*” to the vector but not the virus. It should be widely appreciated that agro-inoculation, which is used more and more for screening, can provide irrelevant data (data that are not applicable, especially in the field) as in this case effective infection/inoculation is dependent on the plant being a host for *Agrobacterium* so that it can deliver the inoculum.

There are specific metrics for indications of “*tolerance*” and “*resistance*” that can be determined. Plant breeders have typically used specific metrics for tolerance and resistance based host/virus systems. “*Tolerance*” can be assumed if there is high titer (as determined by ELISA or other sensitive molecular/biochemical techniques) with negligible symptoms, whereas “*resistance*” is generally defined by an absence of virus and no disease. In reality the titer of virus in a plant can be quite variable and virus titer is not necessarily correlated with disease symptoms. Effects of disease can be gauged by metrics of plant growth and reproduction such as total biomass, yield, plant height, leaf number, seed size, lack of symptoms and frequency of plants in communities. Virus infection/disease can be affected by a number of environmental factors such as irradiance, photoperiod, temperature and thermoperiod.

Susceptibility as determined by measuring phenotypic/biological effects after experimental inoculations can give a false interpretation of natural effects of virus infection. Field based performance is affected by many factors including time of infection (young vs. old plants), uniformity of the infection (are all plants infected at the same time?) homogeneity of the

test population, effective inoculum load, etc. All of these are difficult to assess accurately so as to reflect what happens in the real world.

8. How do environmental or other factors (e.g., temporal variations) affect tolerance and/or resistance? Given the expected variability, what measures of tolerance and/or resistance would be reliable?

Panel Response

Environmental factors, notably the temperature under which diseased plants are grown, have an influence on “*tolerance*” and/or “*resistance*.” In some instances “*resistance*” may be overcome by an increase in temperature. “*Resistance*” of plants obtained by selection or by plant breeding is subject to ‘breakdown’ by viral variants (generated within the plant’s viral population) that can overcome the “*resistance*” and increase the disease. To date, there have not been any instances of ‘breakdowns’ of PVCP-PIPS, suggesting it is a durable resistance strategy.

Most plants do not exhibit virus symptoms; disease is the exception. In many cases, the “*resistant*” plants exhibit no symptoms, but it is also apparent in the relatively few cases that have been investigated, the virus can infect the “*resistant*” plant in the initial cell of entry, but the virus cannot spread from that site, so no disease results (a “*subliminal infection*”). Thus, some “*resistance*” is affected by an inhibition of cell-to-cell movement, rather than a restriction on virus replication *per se*. Environmental factors may affect this mode of “*resistance*”, but it has not been studied extensively. Other plants allow movement of virus from cell to cell, but mount a response that limits the infection to a small area, a local lesion. Elevated temperature can encourage virus movement, and such temperatures may break conventional “*resistance*.” This is particularly evident when “*resistance*” results in a necrotic local lesion. This phenomenon has been well studied, and can result in the systemic movement of the virus. The classic example of this interaction is that between TMV and the N gene of tobacco. Furthermore, when the ambient temperature is reduced subsequently, the whole plant can become necrotic. This “*resistance*” is temperature dependent with “*resistance*” breaking down at elevated temperatures. It is also well known that an abrupt increase in temperature in most organisms leads to a condition called heat shock in which the synthesis of most proteins is shut down. Such a shut down might lead to a breakdown of resistance.

“*Resistance*” to plant viruses generated by plant breeding involving the incorporation of “*resistance*” into crops from other cultivars or species is often not stable because viruses can replicate in such plants (“*resistance*” is often scored as a reduction of symptoms) and there is selection of virulent virus isolates that can overcome the “*resistance*.” Fewer than 10% of the 54 host-virus “*resistance*” gene combinations enumerated by Fraser and Gerwitz (1987) remained effective over a long period. The effect of the environment on this process has not been investigated, although it is probable that environmental conditions that enhance virus replication would increase the probability that “*resistance*”-breaking virus isolates could be induced and selected for.

On the other hand, transgenic coat protein-induced resistance has proven to be very

effective in the most prominent case – *Papaya ringspot potyvirus* (PRSV) resistance in Hawaii. The resistance is viral strain specific but PRSV isolates from other regions of the world could overcome the resistance in laboratory tests. To date, breakdown of resistance based on PVCP-PIPs has not been reported in the field.

The other commercial application of coat protein-mediated resistance is found in virus resistant squash. This is more complicated in that the plants have resistance to three viruses. Thus, some members of the Panel believed that the probability of breakdown of resistance might occur, but the Panel knew of no reports of that happening. The conclusion is that resistance breaking down is a function of changes in the virus, not the plant, in both ‘conventional’ “*resistance*” and coat protein-mediated resistance. Coat protein-mediated resistance so far appears to be stable and reliable.

No member of the Panel could give an example of where the host gene had mutated to breakdown “*resistance*,” nor could they cite examples where changes in other factors such as plant water status, light intensity and salinity resulted in breakdown of “*resistance*.” The Panel was also unaware of any studies on the effects of environmental factors on “*tolerance*.” Experimental evidence of environmental factors that nullify a tolerant phenotype have been obtained in tobacco leaves and in potato tubers (Henderson and Cooper 1977). Temperature can affect “*resistance*” to TMV in some tobacco types, at least for temperature-sensitive isolates of TMV.

Another example of an environmental factor affecting virus “*resistance*” is the potential interaction with non-viral pathogens, where either an increase or decrease in susceptibility to another pathogen is observed in virus-infected plants in comparison to plants not infected by a virus. Examples include the interactions between BYDV and *Erysiphe graminis* (Potter and Jones 1981).

Measures of “*resistance*” are various. For those virus-plant interactions for which a hypersensitive response is responsible for “*resistance*,” the observation, counting and measurement of local lesions is a common measure of “*resistance*.” Obviously, it needs to be carried out under a variety of conditions to obtain confidence that a plant is “*resistant*” under all circumstances that the plant may encounter. For these situations and others, measures of virus accumulation in non-inoculated tissues assess whether the virus has spread through the plant. Similar assays on inoculated tissues address whether there has been replication without systemic movement. These measures include immunological and nucleic acid-based measures. They are generally reliable. But again, a sufficiently wide range of conditions needs to be used for the desired confidence.

9. What would be the ecological significance if a plant population acquired a small increase in viral tolerance and/or resistance above a naturally-occurring level?

Panel Response

Even though introgression happens infrequently, and the consequences of introgression

can be controlled overridingly by genes linked with the transgene, there is a slight chance that a boost in tolerance to a wild plant could be ecologically significant.

Gene flow as defined by the formation of hybrids and backcrossed hybrids is not a risk per se. The ecological consequence of introgression is, in most cases, the over-riding risk factor. There are two helpful ways to think about this risk: How likely is transgene introgression into wild relatives? What would happen if introgression occurred and the transgene provided a small fitness increase (Stewart et al. 2003).

To answer the last question first, since resistance (as defined by the Agency) is an all-or-none phenomenon, there is no such thing as a small increase in resistance. Theoretically, a small boost in viral tolerance or “*resistance*” under constant viral pressure could cause an increase in relative fitness. An increase in fitness would theoretically cause an increase in transgene frequency that would eventually be fixed in a population. This scenario would not necessarily confer increased competitiveness in plant communities. However since there are interrelated ecological factors that control ecological release, it is unlikely a single gene/gene product will be sufficient to change a species’ niche. In addition, this scenario pertains to a directly transformed plant or isogenic line, which begs the question of how likely introgression is to begin with.

Many generations must progress from when a transgene is first introduced from a crop to a near isogenic transgenic wild plant. In general, obligate outcrossers (crop or wild relative) would be expected to have higher gene flow than selfers. In F_1 hybrids, the host genome will contain proportional genomic constituents of the two parents. In BC_1 hybrids, with backcrossing onto the wild plant, and assuming equal sized parental genomes, an average of 25% of the crop genome will be in BC_1 s- along with the transgene. In BC_2 s, 12.5%, BC_3 s will have 6.25% of the crop genome and 93.75% wild genome. While most BC_3 plants will appear very similar to the wild host, they are expected to contain around 2000 crop genes along with the transgene affecting the fitness landscape. Thus, on average, in an advanced transgenic backcrossed plant such as a BC_3 , the transgenic effect can be expected to be swamped by the hitchhiking crop genome effect, thus making it ecologically insignificant (Stewart et al. 2003).

The Agency is interested in predicting the outcome of the introgression of a PVCP-PIP into a wild relative that already has tolerance. If a wild biotype already had a tolerant phenotype, there might not, at first glance be significant ecological effect. The viral load in the host plant might be an important factor to consider. A PCVP-PIP might decrease viral load in the host, which already might be tolerant, but the host could still harbor virus. Virus movement could be a factor to consider.

Biotechnology and cultural conditions can decrease the likelihood of introgression of PVCP-PIPs into a wild relative even further. Some factors could be physical containment, gene use restriction technology, tandem mitigation technologies, and male sterility. In addition, through advanced genomic information, we should be able to determine regions of the genome that are naturally less likely to be introgressed, and these loci would be optimal candidates for transgene integration (Stewart et al. 2003). Natural barriers to introgression will likely be sufficient to disallow persistence of a PCVP transgene in wild relatives.

Based on the hypothesis that concerns about the consequences of gene flow to a wild or weedy relative in the United States may be negligible in certain cases, the Agency is considering whether there are mechanisms to adequately address concerns associated with gene flow so that certain types of VCPs would be of such low risk that they would not need to be regulated by EPA. Below are examples of three conditions (modified from those proposed in 1994) that are intended to significantly reduce any potential adverse effects of gene flow with plants containing a PVCP-PIP.

- (1) The plant into which the PVCP-PIP has been inserted has no wild or weedy relatives in the United States with which it can produce viable hybrids in nature, e.g., corn, tomato, potato, or soybean; or**
- (2) Genetic exchange between the plant into which the PVCP-PIP has been inserted and any existing wild or weedy relatives is substantially reduced by modifying the plant with a scientifically documented method (e.g., through male sterility); or**
- (3) It has been empirically demonstrated that all existing wild or weedy relatives in the United States with which the plant can produce a viable hybrid are tolerant or resistant to the virus from which the coat protein is derived.**

10. Please comment on how necessary and/or sufficient these conditions are to minimize the potential for the PVCP-PIP to harm the environment through gene flow from the plant containing the PVCP-PIP to wild or weedy relatives. Would any other conditions work as well or better?

Panel Response

Overall, Panel members were of the opinion that PVCP-PIPs had no inherent capacity to harm the environment. However, one Panel member expressed concern that in the cases of oat and sorghum, the magnitude and likelihood of gene flow into weedy relatives may be of concern. The Panel accepted that processes aiming at diminished gene exchange are desirable. When setting limits for the sampling of wild relatives for the occurrence of virus tolerance/resistance, the Panel suggested biogeographic regions such as the USDA “hardiness zones”. The Panel was aware of imperfections in the conditions suggested by the Agency but no other conditions were identified as better for the purpose.

The question posed by the Agency presented three scenarios when seeking guidance in helping to define conditions and types of PVCP-PIPs that would not need to be regulated. There was considerable debate about the appropriateness of the implicit assumptions (some Panel members were not convinced of the validity of the assumption that PVCP-PIPs had any capacity to harm the environment).

In regard to condition 1, the Panel was of the opinion that the absence of a competent wild/ weedy relative positioned in relation to the plant containing the PVCP-PIP was an appropriate condition. However this condition should be qualified because different relatives are unlikely to be equally competent as gene recipients.

It was recognized that knowledge of hybridization potential was sparse and of very unequal quality but the likelihood of serious economic harm was such that some plants engineered to contain stress tolerant traits should not be released. In this context, the issue that no virus resistant transgenic oat or sorghum should be released because of the potential risk resulting from gene flow into weedy wild relatives (*Sorghum halapense* and *Avena fatua*) was raised.

When addressing condition 2, the Panel accepted that tactics aiming at diminished gene exchange are highly desirable and even necessary but are not sufficient. None of the current approaches is very efficient and some Panel members subscribed to the view that “if it can happen, it will happen”. Male sterility was explicitly identified in the Agency’s question as an approach to diminished gene exchange but the Panel noted that there might be an unintended impact on wildlife that eat seeds or pollen.

While one Panel member highlighted the potential benefits of gene use restriction technology (GURT), the Panel expressed caution about recombination-mediated approaches (such as CRE/lox integrase or GIN/gix cleavage/ ligation) for there is evidence that geminivirus and pararetrovirus DNA integrate in the germ lines of some plant species (Bejarano et al. 1996; Harper et al. 1999; Hull 1999; Lockhart et al. 2000). Although there are presently known to be only a few examples of this phenomenon, it will be prudent for users of these technologies to investigate genome sequences of plant lines they work with to seek evidence of integrated viral sequences (and avoiding the risky material when it is suspected). Users of such technology would have access to the necessary sequence data and the assessment would not be particularly burdensome.

Condition 3 attracted the attention of the Panel. In their question, the Agency suggested “that all existing wild or weedy relatives in the United States with which the plant can produce a viable hybrid are tolerant or resistant to the virus from which the coat protein is derived” should be identified. It was generally accepted that such wording was not helpful for a number of reasons:

- a) The virus from which the coat protein coding sequence determining the PVCP-PIP was not in all instances the same as that of the target. Thus, LMV may not occur naturally in any *Brassica* crop. Nevertheless, the coat protein from that virus has been used as a source of a transgenic PVCP-PIP in *Brassica napus* where the target is TuMV (Dinant *et al.* 1993).
- b) Not all isolates of a virus infect and cause disease in all plant genotypes and, as a consequence, the unqualified use of the term “virus” when setting a condition for applicants to the Agency was not adequate in this context. It is therefore appropriate in the context of biosafety as well as virus epidemiology to recognize the value of defining specific viral pathotypes or host range variants.
- c) The Panel had particular difficulty when attempting to add precision to approaches that should be followed when sampling wild and weedy relatives for the occurrence of specific virus tolerance or resistance as specified by the Agency. One Panel member favored spreading the

sampling net very wide and suggested that wild relatives of the plant containing the PVCP-PIP under consideration should be sought in all regions of the greater United States (including territories). Another Panel member pointed out that some of these plant populations would be reproductively as well as geographically isolated --and therefore irrelevant. State and county level surveys were briefly considered but were discounted by the Panel because administrative borders do not reflect climate or habitat zones that were key to the sampling of wild communities. Finally, it was accepted that biogeographic regions should be specified and the Panel generally accepted the potential value of using the USDA “hardiness zones” when setting limits for sampling.

Although the Panel was aware of the imperfections in the conditions suggested by the Agency, no other conditions were identified as better for the purpose.

Viral Interactions

Interactions between introduced plant virus sequences and other invading viruses in transgenic plants (e.g., during recombination or heterologous encapsidation) may be a concern to the extent that such events may increase in frequency or be unlike those expected to occur in nature. It has been hypothesized that such events could lead to the creation of viruses with new disease states or transmission properties. The Agency is evaluating the circumstances that might increase the potential for such events to occur and the potential environmental consequences of novel viral interactions in light of the 2000 NRC report which stated that, “[m]ost virus-derived resistance genes are unlikely to present unusual or unmanageable problems that differ from those associated with traditional breeding for virus resistance.” The report went on to suggest that risks might be managed by particular ways of engineering transgenes. However, under either of the 1994 proposed exemptions, the Agency would be unable to ensure that such strategies were implemented. The Agency’s literature review, “Viral interactions in viral coat protein transgenic plants,” discusses possible ways of managing these potential risks in detail.

Viral interactions may occur in natural, mixed infections which are common in plants. Hypothetical concerns related to potential adverse effects resulting from viral interactions between infecting viruses and PVCP-PIPs in transgenic plants may be attributed to opportunities for interactions not expected to occur in nature. EPA is interested in evaluating the significance of novel viral interactions involving a viral transgene.

11. To what extent are novel viral interactions (e.g., recombination, heterologous encapsidation) involving a viral transgene an environmental concern?

Panel Response

Heterologous encapsidation, synergy and recombination are not novel viral interactions as such, since they are phenomena that have been shown to occur in plants infected by more than one virus. The question that was explored by the Panel is whether these interactions could have

novel effects in PVCP-PIPs, compared to their effects in non-transgenic plants. There was good agreement within the Panel that except perhaps for a very few cases, neither heterologous encapsidation nor synergy should be considered to be of serious concern. Although there is no *a priori* evidence that recombination in plants expressing a PVCP-PIP would lead to creation of recombinant genomes that would not also occur in non-transgenic plants, concern was expressed about certain limited situations. The Panel addressed these types of viral interactions separately below.

Heterologous encapsidation. The concern raised is that the transencapsidated virus will have the potential ability to be acquired by a vector with specificity for the transgene PVCP, and that the vector would potentially transfer the virus to a plant not normally infected by the virus. Of course, in the new plant species, the viruses generated will still be the original infecting virus, with its original vector transmission characteristics. Heterologous encapsidation in transgenic plants has a very limited probability of causing interactions different than those that occur in non-transgenic plants infected with more than one virus, and the Panel identified only two situations in which novel interactions could occur.

Heterologous encapsidation could provide a novel interaction in the case where the PVCP transgene is derived from a virus that is not endemic in the region where the infecting virus exists. Generally, the Panel considered there is little justification for use of virus constructs in crops that the virus does not infect, and that constructs from the cognate virus should be preferred. However, there may be cases when using a PVCP gene of a virus that is not present may be useful precisely to prevent the virus from spreading if it is introduced. For instance, if a *Plum pox potyvirus* (PPV) coat protein gene had been introduced into susceptible stone fruit species before the recent introduction of PPV in North America, this could have greatly reduced the impact of the virus on American plum, peach and apricot orchards.

Another scenario in which heterologous encapsidation could produce a novel hazard is when a second vector with a specificity for the new, but not the old plant species picks up the virus and propagates it in the new species and perhaps other species for which it is a vector. This scenario seems unlikely to be novel, since similar events would occur in non-transgenic plants infected by two viruses. Thus, this scenario is likely to have already occurred in the course of viral evolution and should not be considered a palpable hazard.

There are several reasons to consider the degree of potential risks associated with heterologous encapsidation to be relatively slight, and, in addition they can be mitigated. First, in many cases, viruses that are closely enough related for heterologous encapsidation to occur are most often transmitted by the same vectors, and thus the phenomenon would have little or no effect on vector transmission. Second, if unexpected/unwanted effects due to heterologous encapsidation were observed, damage could be limited by simply ceasing to grow the plant variety in question (the problem is reversible). Third, as discussed in response to question 14, there are excellent means of mitigation, which may make it possible to simply render the question moot.

Synergy. Synergy is now understood to be based on viral suppression of RNA silencing. In

using PVCP transgenes, synergy would only rarely be a potential problem, since it is exceptional that a PVCP is responsible for synergy, the only known cases being the coat protein of *Turnip crinkle carmovirus* (Qu et al. 2003; Thomas et al. 2003) and *Cowpea mosaic virus* (Liu et al. 2004). Synergy would also be restricted to the transgenic plant, and is unlikely to have a significantly greater effect than in a mixed infection. Use of PVCP genes that are responsible for synergy with other viruses is not recommended, but in any event farmers would likely soon abandon any transgenic variety that was more susceptible to other viruses.

Recombination. In contrast to heterologous encapsidation and synergy, at least in theory, the impact of recombination could be much greater, since there is now abundant bioinformatic evidence that recombination has indeed, as had been long suspected, played a key role in the emergence of new viruses over evolutionary time.

Thus, viruses do, and will, evolve by recombination, most typically between isolates of the same virus (e.g. Revers, *et al.* 1996). However, recombination between viruses in different taxonomic groups also occurs, and plays a major role in virus evolution when considered in evolutionary time (e.g. Aaziz and Tepfer 1999). Recombination has been demonstrated between viruses and related transgenes (e.g. Allison *et al.* 1996). However, it is important to clarify the misconception that recombination is a rare event. For instance, at the molecular level, there are several experimental systems in which recombination in doubly-infected plants has been observed at high frequency (Aaziz and Tepfer, 1999; Tepfer 2002; Tepfer et al. in review). In addition, in an evolutionary sense, recombination can be considered to be a frequent event. This can be deduced from the fact that viral genomes are assemblies of modules. Each module has its own evolutionary history, often different from that of its neighboring module. As an example, take the protein or proteins required for viruses to move from cell to cell in plants, the movement proteins. One family of such proteins, related to the 30K protein encoded by TMV, serves the movement function in a variety of viruses with entirely different proteins devoted to replication function. They include *Caulimoviridae*, for which reverse transcriptase genes are used for genome replication, *Geminiviridae*, where a rep protein is used, and single-stranded and double-stranded RNA viruses using RNA replicases of multiple superfamilies of RNA polymerases. There is even a 30K family protein in the tospoviruses, which otherwise look like animal bunyaviruses. Looking at genomes from the reverse point of view, there are several closely related virus genera, one of which employs a 30K movement protein and the other employs a triple gene block for the movement function. Similar observations can be made about other modules. Viruses with 30K movement protein genes include isometric and helical viruses. In other words, their capsid protein genes are totally different. And within helical viruses or within isometric viruses, varying movement protein modules may be attached. This clearly shows that there has been a huge amount of scrambling of protein coding modules during the evolution of viruses. Such scrambling obviously implies that recombination has been frequent in the evolution of plant viruses. These studies have demonstrated that while we have the molecular biological tools to detect recombination events, in general we still lack the ability to make informed decisions on their biological significance.

The Panel agreed that the important questions are not the relative likelihood for recombination to occur, but rather whether recombinants in transgenic plants are different from

those in non-transgenic plants and whether they are viable. In order to determine if novel events do occur, the best strategy is to compare recombinants occurring in transgenic and non-transgenic plants. A Panel member gave a progress report on a study of this type. One part of this involved mapping several hundred recombination sites between CMV and a relative, *Tomato aspermy cucumovirus*, across the viral RNA3 in non-transgenic plants (de Wispeleare et al. in press). As expected there are distinct hot spots for recombination, and at the majority of sites precise homologous recombination was observed. However, hot spots for other types of recombination (homologous imprecise and aberrant) were also observed. Similar experiments are under way using transgenic plants, but it is too soon to present detailed results. However, it is striking to note that when the same templates and primers were used *in vitro*, reverse transcriptase produced a remarkably similar assortment of homologous precise recombinants, but none of the imprecise or aberrant sort (Fernandez-Delmond *et al.* 2004). Considering the great biochemical differences between an RNA viral replicase and a reverse transcriptase, this suggests that the determinants for homologous precise recombination would apply to a wide range of situations, and that at least for this type of recombination, nothing novel would be expected in transgenic plants. It is important to note, however, that nonhomologous, similarity-*nonessential* (Nagy and Simon, 1997) recombination represents the most significant tool for emergence of new viruses/strains. No data are currently available on the frequency of nonhomologous recombination in PVCP transgenic versus nontransgenic plants.

Panel members differed concerning the potential biological/epidemiological impact of recombination. Not all recombinants are viable. One Panel member proposed that recombination is not a concern when it involves two viruses (or a virus and a part of a virus, as in PVCP transgenes) that are naturally found coinfecting plants. The hypothesis is that natural selection will have already tested recombinants for viability, and that any hazardous recombinants would already have arisen. Another Panel member responded that this would only be the case if we were in a situation of evolutionary equilibrium, but that this was unlikely because of the constant introduction by humans of new plants, new viruses and new viral vectors in diverse ecosystems around the world. As an example, it was pointed out that at least in the cucumoviruses, recombinants that have a selective advantage relative to the parental strains can be obtained in the laboratory (Fernandez-Cuartero *et al.* 1994; Ding *et al.* 1996). In addition, unpublished work presented by one Panel member showed that one could obtain cucumoviral RNA3 recombinants that induce worse symptoms than the parental viruses. In this particular instance, the recombinant was out-competed by the parentals in co-inoculation experiments. However, these results suggest that there is evolutionary space for a recombinant cucumovirus with properties at least somewhat worse than the presently known viruses.

The Panel members agreed that in most cases there is little *a priori* reason to believe that recombinants between viruses and transgenes will be more of a problem than recombinants between two viruses infecting the same plant, unless transgenes are derived from severe or exotic isolates. The general recommendation to use mild, endemic isolates as the source of the transgene (e.g. Hammond et al. 1999) should minimize any potential for creation of novel isolates that would not equally easily arise in natural mixed infections. The Panel agreed that recombination is a concern when the two contributing viruses have not previously had a chance to recombine. This should be taken into consideration when new viruses are accidentally

introduced into an ecosystem, as is the case of PPV in North America. In addition, there may be considerable economic advantage to creating plants with multiple transgenes that could be deployed in different regions. That one of the viruses contributing transgenes may not be endemic in some regions where multiple coat protein transgenic plants are deployed is a distinct possibility. Here also, the potential impact of recombination should be considered.

12. What conclusions can be drawn as to whether the likelihood of recombination and/or heterologous encapsidation would be increased or decreased in a transgenic plant compared to its non-bioengineered counterpart?

Panel Response

Although unique characteristics of some challenging viruses and viral transgenes may influence recombination frequencies in PVCP-PIPs, the Panel believed that in general recombination was more likely to occur in transgenic plants than in non-bioengineered plants. Whether or not it might lead to viable viruses is unknown. Heterologous encapsidation has been demonstrated in both non-bioengineered plants and transgenic plants expressing functional coat proteins. Heterologous encapsidation should be anticipated for compatible combinations of transgenic coat protein and challenging virus. However, no increase in heterologous recombination or unique combinations of coat protein and viral RNA should be anticipated in PVCP-PIP plants.

Despite aeons of evolutionary refinement and adaptations to their hosts, viruses maintain evolutionary flexibility by preserving their capacity for RNA recombination, high mutation rates through an error prone replicase, and the reassortment of independent genomic components in the case of multicomponent viruses. Comparisons of nucleotide sequences of different viruses have identified segments of similar sequence; these segments are considered evidence of previous recombination events. Such recombination events likely occurred during mixed infections.

Recombination events likely depend on simultaneous replication of two or more viruses in the same cells. This is because simultaneous replication facilitates interaction between the viral RNA templates and heterologous viral replicases, which drive the most frequent recombination events (Nagy and Simon, 1997). Accordingly, recombination events recorded in nucleotide sequences of viruses and examples of heterologous encapsidation suggest that two or more viruses often replicate within the same cell simultaneously. However, this may not be the norm and virus reproductive isolation within a plant may be greater than envisioned. Viruses do not constantly replicate within an infected plant cell, rather the active infection spreads from cell to cell. As replication in one cell is completed, some of the newly replicated RNA is warehoused as virions within that cell and the active infection spreads to adjacent cells where the process is repeated as the infection spreads throughout the plant. Therefore, finding two or more viruses in a plant or a specific plant cell does not necessarily mean that they were introduced to the cell simultaneously or that they replicated in the presence of each other. In fact this may be the exception and a mixed infection may represent a collection of different viruses that were introduced independently and at different times; they may never have replicated simultaneously

in the same cell. Supporting evidence includes inoculations leading to mixed infections of brome mosaic bromovirus and cowpea chlorotic mosaic bromovirus that were most successful when inoculations were separated by two weeks. Simultaneous inoculations lead to the recovery of only one of the two viruses (unpublished). Additionally, Dietrich and Maiss (2003) demonstrated that when plants are infected with two potyviruses, a mutual exclusion phenomenon is observed and only a small proportion of infected cells contain both viruses. Similar results were reported for cucumoviruses (Takeshita et al. 2004). Thus mixed infections may not provide the unlimited RNA recombination opportunities predicted. However, some virus combinations or hosts may accommodate mixed infections and simultaneous replication more readily than others. Estabrook et al. (1998) demonstrated the availability of barley stripe mosaic hordeivirus RNA to *Maize stripe tenuivirus* in *Hordeum vulgare* despite a two week delay in inoculation of the second virus.

In contrast to natural infections, within a PVCP-PIP plant a constitutive promoter ensures that the transcript of a viral transgene is available to a replicating virus in each newly infected cell. Consequently, recombination opportunities are constantly available to a replicating virus and, for many virus combinations, this distinguishes recombination opportunities in PVCPs from those of mixed infections in non-bioengineered plants. Therefore, the likelihood of recombination appears to be increased in transgenic plants as compared to their non-bioengineered counterparts.

Recent experiments designed to detect recombinant cucumoviruses yielded 5-10% recombinants in RT-PCR products from non-bioengineered doubly-infected plants (de Wispeleare et al., in press). Ongoing experiments in the same system indicate that recovery of recombinants involving a cucumovirus coat protein transgenic transcript is significantly higher than in non-bioengineered plants.

Arguments suggesting that recombination in a PVCP-PIP plant may be less frequent than in mixed infections were also acknowledged by the Panel as specific combinations of challenging virus and transgenic transcript may influence recombination possibilities. Such situations include: tissue specificity of the challenging virus or the transgenic transcript may isolate the components from one another; compartmentalization of some viral replication complexes may exclude the transgenic transcript from the replication site; variations in the quantity and stability of the transgenic transcript may reduce its availability for recombination and possible molecular modifications of the transgenic transcript, typical of nuclear generated transcripts may make the transcript an unsuitable template for the viral replication complex.

Heterologous encapsidation of a viral genome depends on specific protein/RNA interactions. The constitutive promoter ensures the availability of coat protein but reports suggest that the quantity is limited as compared to the copious supply of coat protein translated directly from the viral genome. If a viral genome is capable of heterologous encapsidation in mixed infections, it may also occur in transgenic plants. Theoretically, heterologous encapsidation in non-bioengineered plants is dependent on the availability of the coat protein of one virus to the genome of the other, a situation dependent on the simultaneous replication/translation of the two viruses in the same cell. Heterologous encapsidation is now a

recorded event in both non-bioengineered plants and PVCP plants (Farinelli et al. 1992, Fuchs et al. 1999). Since situations may differ for different viruses and in various plant species and transgenic lines, it appears difficult to clearly predict which situation will provide the greater number of heterologous encapsidation events.

Scenarios envisioned involving heterologously encapsidated virus include the transport of a viral genome to a non-host species where it may or may not be capable of replication. Even if replication does occur, the genome no longer has access to the transgenic derived coat protein and must be encapsidated in its own coat protein. Therefore, no real change has occurred other than the virus is now marooned in a new host without access to its adapted vector. There remains the possibility that predators of this plant species are unique to the virus but are suitable vectors for its distribution. In this case the plant could serve as an intermediate host and a bridge for the virus to move to yet another plant species. This scenario is considered unlikely. The general consensus of the Panel was that heterologous encapsidation in PVCPs is of little consequence for any viral genome escaping the PVCP in a foreign coat protein as further access to the unique transporting coat protein structure would be unavailable in the new host.

A number of methods for reducing the frequency of recombination and heterologous encapsidation have been identified. While the effectiveness of these techniques has been verified for particular cases, their applicability to all PVCP-PIPs is unclear. Recognizing that it would be difficult for a product developer to measure rates of recombination, heterologous encapsidation, or vector transmission under field conditions, EPA is considering whether it would be necessary to verify that such methods worked in any particular instance by measuring rates in modified versus unmodified plants.

13. How effective is deleting the 3' untranslated region of the PVCP gene as a method for reducing the frequency of recombination in the region of the PVCP gene? Is this method universally applicable to all potential PVCP-PIP constructs? Would any other methods work as well or better? Which methods are sufficiently effective and reproducible such that actual measurement of rates to verify rate reduction would be unnecessary?

Panel Response

The Panel agreed that deleting the 3' UTR may reduce the frequency of recombination in some but not all viral systems. Whenever possible, other methods should be considered to decrease the recombination frequency, and a combination of several methods was recommended. However, none of these methods is sufficiently effective to eliminate recombinants. Thus, the surveillance of recombination events might be necessary. It should be noted that several Panel members believed that the question posed by the Agency was irrelevant since they did not believe that these three processes needed measurement for PVCP-PIPs, arguing that these processes did not pose significant risks.

The first question posed by the Agency dealt with how effective deleting the 3' untranslated region (3' UTR) of the PVCP gene is in reducing the frequency of recombination. The Panel agreed that deleting the 3' UTR should decrease recognition of PVCP mRNA by viral

replicase and thus decrease RNA recombination. It is known that replication enhances the frequency of viral RNA recombination, not only because replication propagates and increases the level of recombining RNA substrates but also due to the mechanism of recombination. Template switching by the replicase enzyme is a commonly accepted mechanism of viral RNA recombination, and in many viruses 3'UTRs carry the initiation promoters of RNA replication. In addition, deleting the 3'UTR may destabilize the transgene mRNA.

Regarding the second question, the Agency wanted to know if the removal of the 3' UTR was universally applicable to all potential PVCP-PIP constructs. The Panel recognized that deleting the 3' UTR may be effective to a different degree for different viruses. This is because some viruses carry the replication signals in other locations that are distant to the 3' UTR. It has been demonstrated that genomic or subgenomic promoter regions can per se serve as recombination hot spots, e.g. by studying recombination mechanisms in a model bromovirus system (Olsthoorn et al., 2002; Wierzchoslawski et al. 2004). In such cases, in addition to the 3' UTRs, the remaining active regions (genomic and subgenomic promoters, replicase binding sites) must be deleted from the transgene PVCP mRNA to secure the greatest possible inhibition of RNA recombination. Generally, it is expected that the removal of the active regions will prevent direct initiation of RNA replication on the PVCP RNA template and then double recombination (crossover) events will be required to secure recombination. However, the requirement for double recombination events drastically reduces the chance for viruses to generate a new functional recombinant virus.

This method is not universally applicable to all potential PVCP-PIP constructs. Deleting the 3' untranslated region of the PVCP gene is only important for those viruses in which this region contains the promoter for minus strand synthesis. A more appropriate statement would be that all promoters, minus strand, plus strand and other cis-acting replication elements should be avoided in such constructs.

The 3' (UTR) has been maintained in genetic constructs partly because it is believed to provide stability to the RNA transcript within the cytoplasm, due to their typical secondary structure, and partly because they were associated with the original clone, as numerous cDNA clones used to establish coat protein mediated resistance used primers that hybridized to the 3' terminus of the virus. This is especially true of polyadenylated viral RNAs. Due to the biochemical rules of DNA and RNA replication, the replication process is initiated at the 3' end of the virus utilizing a sequence/structure maintained in this 3' UTR. Thus by including the 3' UTR in the construct, a related virus may recognize the viral transcript as a template and initiate replication on the transgene transcript. Two things may happen. The replication complex may use this initiation point to begin replication and switch back to the viral RNA as a template during recombination or the replication complex may make a complementary copy of the viral transgene which may also be available for RNA recombination. Since all RNA viruses must use 3' UTRs as replication initiation sites, it makes good sense to withhold these sites from viral transgenes as it would appear that they enhance recombination activity of the transgenic transcript. Also, by deleting the 3' UTR double recombination events would be required, which is less likely to occur. In laboratory experiments where transgenic constructs have been involved in recombination events, removal or disruption of 3' UTRs have reduced recombination to below

experimental detection limits. If transcript stability becomes an issue, other non viral RNA stabilizing sequences could be added.

The third question dealt with other methods that will work as well or better than removal of 3' UTR. The Panel recognized that other (supplementary) methods of alleviating the risk of recombination include the reduction in sequence homology between PVCP and viral RNAs (e.g. by changing the third base in the codon triplet), or changing, whenever possible, the sequence composition (GC versus AU) without major alterations in the translated amino acid sequence. This is because AU-rich sequences could serve as recombination hotspots for several RNA viruses (Shapka and Nagy 2004; Nagy et al. 1999).

The fourth question asked by the Agency addressed which methods are sufficiently effective such that requiring the measurement of recombination rates would be unnecessary. The Panel doubted if the above methods are sufficiently effective to warrant the reduction of recombination rates below the level that the actual measurement will be unnecessary. The Panel presented several points to support their position. First, each particular viral system responds differently to the described preventive approaches, as each virus has its own set of tools governing molecular interactions during RNA-RNA recombination. Second, although unproven, recombination may depend upon host factors so the same virus may recombine at different rates in different plants. Third, there might be mechanisms other than copy choice supporting viral RNA recombination, e.g. breakage-and-religation. For the latter, the removal of replication signals might not help to completely eliminate the religation events.

In addition to removal or disrupting the 3' UTR, several other methods have been suggested for reduction of recombination events involving the transgene. These include using the smallest resistance-generating viral fragment possible to generate resistance, disrupting potential AT rich recombination hotspots, providing point mutations and/or deletions in the transgene that would disable a functional aspect of the coat protein, when identified. These methods can be combined to help ensure that either recombination does not occur or that the incorporated segment is useless to the recombinant.

14. Are any methods for inhibiting heterologous encapsidation or transmission by insect vectors universally applicable to all PVCP-PIPs? Which methods are sufficiently effective and reproducible such that actual measurement of rates to verify rate reduction would be unnecessary?

Panel Response

The Panel agreed that universally applicable methods were not available for inhibiting heterologous encapsidation or insect vector transmission, but that methods were generally available on a case-by-case basis if either of the above-mentioned events posed a real risk.

The first question links two things, heterologous encapsidation and vector transmission, which are not necessarily synonymous. Encapsidation is a pre-requisite for vector transmission, but it is important to realize that encapsidation does not always result in vector transmission.

Strictly considering heterologous encapsidation, it should be possible to delete, or mutate amino acids known to be important in virion formation/assembly, but for many viruses these targets are as yet unknown. We do know some of the amino acids that are essential for capsid formation in specific viruses, for example the amino acids (S, R, D) for capsid assembly in viruses having filamentous virions are conserved among these viruses. These could be targeted for affecting encapsidation, but whether or not protection would remain is a question. Varrelmann and Maiss (2000) have demonstrated for PPV that mutation of the PPV coat protein prevent heterologous encapsidation while maintaining resistance. However, in at least one example (TMV), the ability to form virions is also an important component of the protection.

Regarding vector transmission, in general, restricting the portion of the PVCP transgene to that encoding the structurally essential core may be a somewhat universal strategy for limiting potential adverse effects (i.e. vector transmissibility) of heterologous encapsidation, but not for limiting heterologous encapsidation itself. For isometric viruses, only the S domains would be used, omitting the P domains that interact with other organisms. For helical viruses, maintaining the four helix bundle core and the internal, nucleic acid binding, loops while dispensing with the surface displayed N-terminal and C-terminal extensions would reduce the chances of unwanted vector transmission, but still likely retaining effectiveness as a plant protectant. In some instances where important determinants are more well known, it should be possible to alter the specific amino acids which are known to confer known important biological properties: the DAG in the amino-terminal region of the coat protein of potyviruses for aphid-vector transmission; the RT domain for luteoviruses, and various other protruding or outer capsid domains for different viruses (CMV, fungal-transmitted tobravirus). However, there was some discussion regarding demonstrated effectiveness of these strategies in transgenic plants. Nevertheless, if successful, the strategy of targeting adverse effects of heterologous encapsidation may be more possible than modifying coat protein genes to strictly prevent encapsidation.

Both heterologous encapsidation and vector transmission could also be reduced by lowering transgene expression levels, or instead of expressing full-length coat protein genes that yield coat protein molecules, constructs could be designed instead to confer resistance via post-transcriptional gene silencing. The lowest concentration consistent with protection could be used to reduce frequencies of heterologous encapsidation and recombination.

15. How technically feasible would it be to measure rates of recombination, heterologous encapsidation, and vector transmission in PVCP-PIP transgenic plants in order to show that rates are reduced?

Panel Response

For all three aspects of the question (recombination, heterologous encapsidation and vector transmission), it is technically feasible, though often challenging, to test whether modification of the PVCP gene reduces these processes. In assays for all three processes, Panel members believed that one of the most challenging tasks was the design, execution and validation of appropriate controls for the assays.

Recombination rates. Recombination rate is defined as the number of recombination events occurring per unit of time. The virologically meaningful unit of time is a replication cycle. Unfortunately, we do not have accurate numbers for how many replication cycles occur between initial infection of a plant and its harvest. The number depends on how many cells are on the pathway that the infection takes, whether a new replication cycle must occur in every cell in the pathway and whether multiple cycles occur in a single cell. These factors likely are different for different viruses.

Fortunately, there is another quantity that is easier to measure and directly related to recombination rate. That is the frequency of recombinants. The rate of recombination is equal to the frequency of recombinants divided by the number of replication cycles (or some other measure of time). Since the question implies an interest in whether recombination rates are reduced, it can be answered confidently if the frequency of recombinants is reduced. Comparison between an unmodified PVCP transgene and a PVCP transgene modified to address some of the concerns are dealt with elsewhere in this discussion.

It is theoretically technically feasible to measure the frequency of recombinants in a population of viruses, although in practice the approaches used are difficult to implement. One popular approach is to create multiple molecular clones of a region of the viral genome in which the recombination may have happened and determine their nucleotide sequences to score whether or not they are recombinants. A large number of clones are required to obtain statistically valid results. However, with current costs of nucleotide sequencing, such an undertaking is not cost prohibitive.

A second approach is allele specific real-time polymerase chain reaction (PCR). In this approach, two pairs of primers are designed, one pair in which each primer will only prime from the genome of the infecting virus and the other pair in which each primer will only prime from the transgene. Three or four PCR reactions are carried out: those with each pair as described serve as controls to measure the amounts of non-recombinant sequences; and those in which pairs exchange partners, allowing amplification of reciprocal recombinants. When the PCRs are performed in real-time mode, reliable estimates of the amounts of each template present are obtained. The frequency of recombinants is the quotient of the sum of the exchanged values over the sum of all values.

A third approach is possible if the sequences differ in multiple restriction endonuclease recognition sites. Restriction will generate unique recombinant DNA fragments whose quantity can be determined by densitometry of a gel electrophoretic separation of the digestion products. Comparison with the quantities of the corresponding non-recombinant fragments will result in a value for the frequency of recombinants.

One member of the Panel with experience in conducting measurements of recombination explained that, in practice, approaches to measuring frequencies of recombinants are difficult to implement. The greatest success was achieved by employing PCR based methods (Dardick et al. 1999).

The above approaches were offered assuming that the Agency was interested in recombination between the transgene and the nucleic acid of a second invading virus. An approach valid for determining whether recombinant frequencies were reduced when recombination between two inoculated nucleic acids occurred in a PVCP-PIP transgenic plant relative to a non-transgenic plant was also offered. This approach was to combine two RNA transcripts, one containing the 5' end of a viral genome through the coat protein gene, the other containing the section including the coat protein gene through the 3' end of the viral genome. Recombination should result in infectious RNA that can be assayed by any method for detecting infectious particles, such as local lesion assay on an appropriate indicator host.

Transencapsidation. Relative to transencapsidation, the rate concept has even less relevance than for recombination. For transencapsidation to be a problem the rate needs to be close to the rate for cisencapsidation, otherwise there will be no transencapsidated virions. As with recombination, the frequency of transencapsidated genomes can be a useful measure for the potential of transencapsidation.

Fortunately, the frequency of transencapsidated genomes can be measured by one of four methods. Immunocapture PCR or RT-PCR in real-time format should reveal how much of the viral nucleic acid target of the reactions is encapsidated in particles that contain at least some of the PVCP that is the target of the antibody used. An antibody against the transgene PVCP is used to separate virions that bear subunits from the transgene from those that do not. The nucleic acid in them is released and quantified by quantitative PCR or quantitative RT-PCR as appropriate. Concurrently, the total virion population is also subjected to release and quantitative PCR or RT-PCR. The value serves as the denominator in the quotient that gives the frequency of transencapsidated genomes.

Immunoelectron microscopy can serve a similar purpose. In a plant bearing a PVCP transgene, the only genome encapsidated can be assumed to be that of an invading virus. The question then becomes what proportion of the virions formed have a capsid that is composed in part of subunits provided by the transgene. Staining of virions with tagged (for example by ferritin) antibodies against the transgene PVCP should allow distinction of stained virions from unstained ones. A difficulty with this approach is that only a small percentage of capsid subunits usually react in such studies. When only a small percentage of the subunits are derived from the transgene, many virion particles may escape scoring as being transencapsidated. However, since the aim of the assay is to test for reduction of the frequency of transencapsidated genomes, the underestimation resulting from this problem is minimized, as long as there is appreciable evidence of transencapsidation in the case of the non-modified PVCP transgene. This method was criticized based on practical experience of one Panel member who noted that the variation in the density of transgene PVCP on virion particles varied widely and unpredictably. The Panel member felt that, as a result, this was not an assay that could lend itself easily to measuring the extent of reduction of transencapsidation by modification of the PVCP-PIP.

The methods favored by Panel members who have assayed transencapsidation were western blotting and ELISA. In the western approach, virions are purified. The proteins in the

virions are separated by SDS-PAGE and transferred to membranes. One membrane is probed with a monoclonal antibody (mAb) against the normal PVCP, the other with a mAb to the transgene PVCP. In the ELISA approach, the virion preparation is tested by ELISA for the amount of each of the two PVCPs. In either case, observing how the ratio between the two PVCPs changes with modifications of the PIP will reveal whether transencapsidation has been reduced as a result of the modification. These assays address what percentage of the capsid proteins in virions come from the transgene, while the previous two assays determine what percentage of the virions contain a PVCP from the transgene, perhaps a more relevant quantity for considerations of potential transmission.

Vector transmission. Vector transmission plays a role in two scenarios. When PVCP transgenes are used as PIP, it is often the case that the protection afforded the plant is not absolute. Low levels of the virus may result or the plant may eventually escape from protection. The second scenario occurs when infection is by a non-targeted virus and transencapsidation has occurred.

Vector transmission assays are diverse because of the diversity of organisms that vector plant viruses: arthropods, fungi, nematodes, and mammals (mechanical transmission through contact). If the virus vector is well understood, there usually are assays available for measuring the efficiency of vector transmission. For example, in the case of arthropods, the experiments involve caging the arthropods with diseased plants for a specified period of time (acquisition access period) and then transferring them to healthy test plants for a second period of time (inoculation access period). For viruses whose transmission mode is circulative and /or propagative, an additional period of feeding on healthy plants intervenes to allow the virus to circulate and replicate in the arthropod. Scored are the percentages of test plants that become infected. These tests are standard in vector entomology. For soil borne vectors such as fungi and nematodes, transmission tests are also available, but are somewhat more difficult to execute.

Thus, for all three aspects of the question, it is technically feasible, though often challenging, to test whether modification of the PVCP gene reduces recombination, transencapsidation and vector transmission. In assays for all three processes, Panel members concluded that one of the most challenging tasks was the design, execution and validation of appropriate controls for the assays. It should be noted that several Panel members felt that the question posed by the Agency was irrelevant since they did not believe that these three processes needed reduction for PVCP-PIPs, arguing that these processes did not pose significant risks.

EPA recognizes that scientific disagreement exists as to the likelihood of environmental impacts due to novel viral interactions in transgenic plants modified with PVCP-PIPs. The Agency is considering whether there are available mechanisms to adequately address concerns associated with novel viral interactions so that certain types of PVCP-PIPs would be of such low risk that they would not need to be regulated by EPA. Below are examples of conditions that might significantly reduce either the novelty [(1) and (2)] or frequency [(3) and (4)] of viral interactions in PVCP-PIP transgenic plants.

(1) The genetic material of the PVCP-PIP is translated and/or transcribed in the same cells, tissues, and developmental stages naturally infected by every virus from which any

segment of a coat protein gene used in the PVCP-PIP was derived.

(2) The genetic material of the PVCP-PIP contains coat protein genes or segments of coat protein genes from viruses established throughout the regions where the crop is planted in the United States and that naturally infect the crop into which the genes have been inserted.

(3) The PVCP-PIP has been modified by a method scientifically documented to minimize recombination, (e.g., deletion of the 3' untranslated region of the coat protein gene).

(4) The PVCP-PIP has been modified by a method scientifically documented to minimize heterologous encapsidation or vector transmission, or there is minimal potential for heterologous encapsidation because no protein from the introduced PVCP-PIP is produced in the transgenic plant or this virus does not participate in heterologous encapsidation in nature.

16. Please comment on how necessary and/or sufficient each of these conditions is to minimize the potential for novel viral interactions. Please address specifically what combination would be most effective or what conditions could be modified, added, or deleted to ensure that potential consequences of novel viral interactions in PVCP-PIP transgenic plants are minimized.

Panel Response

These four strategies for reducing risk of novel virus interactions are of modest value as indicated individually below. Two types of objections to these four conditions were raised. One was based on the relatively ineffective nature of these measures. If one really wishes to eliminate novel interactions, then resistance based on post-transcriptional gene silencing is an obvious solution. However, the Panel was aware that this approach was not a subject for consideration. The second type of objection, raised by several Panel members, was that the potential novel interactions are not of serious concern, and thus did not merit that such measures be taken.

(1) The genetic material of the PVCP-PIP is translated and/or transcribed in the same cells, tissues, and developmental stages naturally infected by every virus from which any segment of a coat protein gene used in the PVCP-PIP was derived.

Most plant viruses are present in a wide range of cell and tissue types. This question is only pertinent regarding the viruses where this is not the case, such as those limited to phloem tissue. The advantage of limiting transgene expression to only phloem cells was criticized for several reasons. Even phloem-limited viruses escape into other tissues when certain other viruses are present, and in any case, phloem-specific promoters are far from totally specific. So this idea would be of rather limited effectiveness, and in fact the situation it is designed to prevent does already occur in nature. Thus, several Panel members considered that plants expressing a PVCP-PIP would not represent novel situations, even regarding phloem-limited viruses.

(2) The genetic material of the PVCP-PIP contains coat protein genes or segments of coat

protein genes from viruses established throughout the regions where the crop is planted in the United States and that naturally infect the crop into which the genes have been inserted.

What is described here is most often implemented: in designing a PVCP transgene, better efficacy is often observed if it is as similar as possible to the target virus. Most often similar virus strains/isolates are present quite broadly. This question is only pertinent for cases when the coat protein gene of a virus absent from the area is introduced into plants in order to prevent a possible introduction of the virus from having a major disease impact. Since using such an exotic PVCP gene would open possibilities for novel interactions, these cases would need to be studied individually.

(3) The PVCP-PIP has been modified by a method scientifically documented to minimize recombination, (e.g., deletion of the 3' untranslated region of the coat protein gene).

As described in response to question 13, methods for minimizing recombination are only partially effective. For this reason, the question remains whether novel recombinants would be created in transgenic plants, and simply reducing the frequency of these events is not an answer to the question.

(4) The PVCP-PIP has been modified by a method scientifically documented to minimize heterologous encapsidation or vector transmission, or there is minimal potential for heterologous encapsidation because no protein from the introduced PVCP-PIP is produced in the transgenic plant or this virus does not participate in heterologous encapsidation in nature.

Methods for reducing heterologous encapsidation or vector transmission are in contrast to recombination and as described in response to question 14, potentially very effective. This method can thus be considered seriously if deemed necessary.

Other Questions

In 1994 EPA proposed exempting plant viral coat proteins from the requirement of a food tolerance under the Federal Food, Drug, and Cosmetic Act based on rationale that (1) virus infected plants have always been a part of the human and domestic animal food supply and (2) plant viruses have never been shown to be infectious to humans or mammals. The safety of consuming plant virus genes has been supported by experimental work (Chen et al. 2003; Rogan et al. 2000; Shinmoto et al. 1995) and expert consultations including the 2000 NRC report which concluded that, “viral coat proteins in transgenic pest-protected plants are not expected to jeopardize human health because consumers already ingest these compounds in nontransgenic food.” However, EPA recognizes that PVCP-PIP developers may wish to modify the PVCP-PIP construct and that some methods of mitigating potential risks associated with recombination and heterologous encapsidation might actually require them to do so. Such modifications might result in changes to the protein(s) produced thus creating potential food safety concerns, e.g., inadvertent production of new toxins or allergens (Day 1996). Modifications of the construct and alteration of the proteins produced creates the potential for health impacts on non-target species as well as humans.

17. To what degree and in what ways might a PVCP gene be modified (e.g., through truncations, deletions, insertions, or point mutations) while still retaining scientific support for the idea that humans have consumed the products of such genes for generations and that such products therefore present no new dietary exposures?

Panel Response

The Panel agreed that (because of the human history of consuming virus infected food), unaltered PVCPs do not present new dietary exposures. There was no clear consensus on how much change would be necessary to invalidate this assumption, although there was general agreement that the appropriate comparison is to the range of natural variation in the virus population.

As a point of initial context, the Panel recognized that only expressed protein products are of concern for human health. Situations involving only the production of nucleic acids do not pose any concern. However, it was suggested that the actual product produced in a modified plant needs to be characterized to insure that read-through proteins are not produced.

The question as posed by the Agency is based on the generic assumption that PVCPs are safe because there is a history of safe consumption. The Agency also cites some literature and previous expert consultations in support of this assumption. One Panel member cited personal experience in support of this assumption, stating that a large number of viruses are easily isolated from produce purchased from the supermarket, including from store-bought tomatoes, peppers, cucurbits, potatoes, asparagus, celery, and other store-bought produce. The one concern that was raised in regard to this assumption related to generalization to all virus families from the specific examples given. History has shown that occasionally the wide spread consumption of foods that have previously been rare can have unexpected consequences; such as the occurrence of kiwi allergies.

The question posed by the Agency asks how much change can be introduced before the “safe history” assumption is no longer valid. In this context, only changes that affect an expressed protein are of concern. Changes to regulatory and untranslated regions are not relevant. The answer to this question needs to be considered in relation to the natural variation for each individual virus. How much variation occurs in the natural population? What are the relative frequencies of point mutations versus insertions/deletions? Are there hot spots for each type of change in the sequence? In other words, the correct question may not be how much an individual protein has been changed, but how that change relates to the range of alleles seen in the population. In many cases, there are data showing a high degree of variability in amino acid sequence between the PVCPs of different isolates of the same virus – in the case of potyviruses this is especially true within the N-terminus, as seen with PPV, PRSV and TuMV (Shukla et al. 1994). In this case, anything less than a major mutation is thus unlikely to differ significantly from the variability extant in viral populations. In general, changes that can be considered to be within the bounds normally found in the viral population can probably be considered to be as safe as the initial PVCP.

For changes that fall outside this range, the Panel suggested that there are three potential health affects that might be of concern: (1) direct toxicity, (2) allergenicity, and (3) PVCP-induced changes in plant cell metabolic regulatory networks resulting in production of harmful metabolites. The Panel was not aware of any examples where a plant PVCP is known to be a human toxin. Several Panel members suggested that it is difficult to see how changes that result in toxicity could be made unknowingly since most forms of protein toxicity involve specific interactions. On the other hand, one Panel member stated that many PVCPS have domains on their external surfaces that appear not to play direct roles in genome encapsidation. Indeed, a variety of additional functions have been attributed to PVCPS, including intracellular and intercellular and long-distance transport and targeting. Alteration of the VCP sequence may modify such functions, and other functions yet to be discovered, so that they would perform toxic functions if they were to enter into body cells. The potential exists for coat proteins to resist digestion in the human alimentary tract. Beyond such survival, attachment and/or entry into body cells is necessary for an enzyme to perform as a toxic catalyst. Some, but not all, PVCPS have domains that are responsible, either alone or in concert with other proteins, for the attachment to arthropod cells. The attachment serves the virus in transmission, allowing a vector to acquire and deliver the virus to a new plant. The structural transition required to change from binding to vector cells to binding to human cells may not be a large one.

Allergenicity is a more difficult issue because it is still not clear why only a few of the thousands of food proteins that are consumed each day become allergens. Searches of several allergen databases failed to find any viral proteins that have been identified as allergens. Anecdotal evidence for the relative lack of allergenicity for PVCPS is also suggested by the observation that several virologists at Rothamsted (Kassanis, Bawden, and others) who had handled (and mouth-pipetted) many purified virus preparations over many years collected their own blood and assayed it for antibodies against any of the common viruses which they had used; no virus-specific antibodies were found. In addition, several plant viruses are being used as carriers for epitope presentation in research on edible vaccines. This work has shown good immunological response to the target epitope. Re-immunization of the same individuals with distinct vaccines based on the same carrier plant PVCP resulted in production of antibody to the vaccine epitope without interference from a response to the carrier VCP molecule. This suggests that sensitization to the PVCP is not likely to be a problem (V. Yusibov, personal communication to J. Hammond). One Panel member suggested that both cell-mediated and IgE-mediated immune responses should be considered, although little is known about the former. Several Panel members pointed out that allergenicity relates to the three dimensional shape of the protein. Many Panel members believed that it would be appropriate to ask a developer to apply the same allergenicity assessment procedures that are used for other PIPs to highly modified PVCPS to provide some assurance that there are no potential cross-reactive sequences. Structural analysis might also be carried out as the data bases on allergen structures improve. One Panel member also raised the possibility that such analysis might also avoid situations where pollen-borne PVCPS might act as inhalant allergens.

Further, it is generally accepted that the level of exposure plays a role in allergenic sensitization. The Panel agreed that, as long as bioengineered PVCPS are expressed at levels

significantly below those naturally found in plants, sensitization seems unlikely.

The possibility that a modified PVCP might interact with the regulatory machinery of the plant, altering it in such a way that the plant now produces substances harmful to human health, was also raised. Plant viruses already interact with the plant regulatory machinery. Alterations are often profound and lead to what we observe as symptoms of disease. Regulatory networks are just now beginning to be unraveled. Experiments to date have revealed many unexpected associations; there will likely be more. At this stage, we can not rule out changes that may lead to production of harmful substances. The probability of such changes is probably greatest for plants for which human experience has already taught that consumption of some parts of the plant or of the plant at certain stages of growth is dangerous. We eat rhubarb stems, not leaves. Castor bean seeds are toxic, but castor oil is a good source of certain vitamins.

Finally, it was suggested that another reason to catalogue the degree of variability found in natural virus populations is to estimate how much a PVCP can be modified and still be functional in the context of disease reduction. Given the various structural interactions involved in VCP function, it is likely that a modified protein will prove to be ineffective in conferring '*resistance*' before the changes raise human health issues.

Overall, the Panel agreed that (because of the history of consumption), unaltered PVCPs do not present new dietary exposures. Some Panel members felt that a potential allergenicity analysis would be appropriate for all single amino acid substitutions, while others felt that this would only be appropriate if the single amino acid substitutions fall outside the normal range of variation for a particular virus. There was general agreement that an allergenicity assessment would be appropriate for insertions or deletions, except perhaps for terminal deletions that do not affect overall protein structure.

18. What are the potential adverse effects, if any, of such modifications on nontarget species (e.g., wildlife and insects that consume the PVCP-PIP)?

Panel Response

The Panel agreed that virus-protected crops should not be as attractive to aphids as diseased crops and thus may reduce the numbers and types of organisms that spend at least part of their life cycles in specific agroecosystems. However, because many agroecosystems are transient in nature, the impact of any changes in numbers of aphids in PVCP-PIP plants should be negligible over larger scales such as the landscape. Additionally, the Panel was not aware of any risk posed by PVCP-PIP crops to wildlife.

Potential adverse effects in wildlife can be manifested as direct and indirect effects. Direct effects are effects that occur in an organism exposed directly to a potential toxicant. Indirect effects are effects on organisms that have not been exposed to a toxicant but these organisms are part of a food web that interact with organisms that have been exposed. Thus, indirect effects occur when changes in populations of exposed organisms result in changes in populations of unexposed organisms. Direct effects can be further divided into lethal and

sublethal effects (Stark et al. 2004). Mortality, the lethal effect, may occur rapidly or may be delayed. Sublethal effects include but are not restricted to: reductions in life span, reduction in the numbers of viable progeny, failure to reach optimal weight, delays in the time to first reproduction, genetic mutation of gametes, tumors –including cancers, changes in behavior resulting in less competitiveness for food, mates and the ability to avoid predators. Multiple sublethal effects may be manifested in an organism after exposure to a toxicant (Stark and Banks 2003).

Lethal effects in animal life after feeding on PVCP-PIP plants are highly unlikely because plant viruses are not known to have deleterious effects on animal life. Additionally, animals routinely feed on non-engineered virus-infected plants and do not die. If animals did die after ingestion of virus-infected plants, then these viruses would be developed as insecticides, rodenticides, molluscicides, etc. Production of other toxic substances such as an increase in secondary plant metabolites in response to a PVCP-PIP may be a possibility and result in toxicity. However, this scenario is also improbable.

Sublethal effects after feeding on PVCP-PIP plants may occur if for example, nutritional changes within the plant occur due to a trade-off for having additional viral genes. There may also be some subtle mechanism of toxicity that has not been defined to date such as toxicity of specific viral proteins as well as production of other toxic substances in response to PVCP-PIP. Nevertheless, sublethal effects are not expected to be manifested in animal life, again because wildlife and insects regularly feed on non-engineered virus-infected plants with no apparent sublethal damage.

Induction of the Hypersensitive Response as a consequence of coat protein modification to disrupt subunit:subunit interactions would probably result in loss of plant productivity – and adverse effects for the producer as well as possible limitation of food resources for wildlife and other non-target species. Such lines would either not be brought to deployment, or would rapidly be withdrawn.

Indirect effects are very unpredictable and cannot be entirely ruled out. Examples of indirect effects in other types of genetically modified crops have been reported, in particular for Bt engineered crops and herbicide resistant crops. For example, aphids are often more attracted to diseased than healthy plants. This in turn should result in increases in predators and parasitoids of aphids and changes in the numbers and perhaps the types of other organisms that visit or develop in agroecosystems, thus resulting in changes to food webs. Virus-protected crops should not be as attractive to aphids and thus may reduce the numbers and types of organisms that spend at least part of their life cycles in specific agroecosystems. However, because many agroecosystems are transient in nature, the impact of changes in numbers of aphids in PVCP-PIP plants should be negligible over larger scales such as the landscape.

Modifications of the construct may also potentially create the opportunity for novel viral interactions because the inserted virus sequences could be unlike any that occur naturally.

19. To what degree and in what ways might a PVCP gene be modified (e.g., through truncations, deletions, insertions, or point mutations) before it would be a concern that novel viral interactions due to the modifications could occur because the PVCP gene would be significantly different from any existing in nature?

Panel Response

Modifications that fall within the natural variability of the PVCP were considered to be of little concern by the Panel. More important changes would be more likely to reduce the effectiveness of the PCVP than to cause new interactions to occur. Some concern regarding insertion into the PVCP gene was expressed.

It should be pointed out that RNA viruses are thought to lack proofreading by their replicases, and thus the population of viral genomes within an infected individual is structured in a quasispecies, i.e. as a cloud of variants of a consensus/master sequence. Concretely, when one simply sequences a number of cDNA clones from an infected individual, a rich assortment of modifications is observed, including ones that are certainly non-viable, but the modified genomes are presumably replicated *in trans*. These include deletions, insertions, and point mutations. When comparing related viruses, the array of possible variants is also quite broad. There is significant variability between isolates of any particular virus, which is reflected in antigenic variability, and for potyviruses there is considerable variation in length and sequence of the N-terminal domain of the CP. If the modifications made in a PVCP gene go beyond what is naturally occurring, one would predict that these modified proteins would be that much less likely to be involved in viral interactions, and that much less a reason for concern. In addition, excessive introduced variability intended to ameliorate potential risks might have a greater probability of reducing the efficacy of the "resistance" conferred by the PVCP-PIP gene than of resulting in novel viral interactions.

Truncations, deletions or point mutations are unlikely to cause concerns about novel viral interaction. In contrast, if insertions are made in the PVCP gene, this could have significant effects, of which two were mentioned by Panel members. Chimeric PVCP genes could be created either intentionally or by readthrough from the transgene into plant sequences. This should be avoided, since the protein could possibly have novel properties. It would also be important to avoid modifications that might increase the probability of recombination with other viruses, such as inclusion of a 3' non-coding region from a heterologous virus.

The potential risk issues identified by this paper are specific to virus-resistant transgenic plants. However, the Agency recognized that it may be necessary to evaluate other information related to the PVCP-PIP.

20. Would any additional requirements related to PVCP-PIP identity and composition (e.g., demonstration that the transgene has been stably inserted) be needed for significant reduction of risks associated with PVCP-PIPs?

Panel Response

The Panel could not identify any additional requirements related to PVCP-PIP identity and composition. The example given in the charge to the Panel (demonstration that the transgene has been stably inserted) was considered to be of no consequence and not to be an issue. In the transformation process, many plants are produced, and then they are tested for the desired trait, saving only those that retain the desired trait (in this case resistance to the virus) over a number of generations. Aberrant transformants are ones with unstable expression of the transgene and would be discarded early in product development.

21. Are there any considerations beyond gene flow, recombination, and heterologous encapsidation as posed in the preceding questions that the Agency should consider in evaluating the risk potential of PVCP-PIPs (e.g., synergy)?

Panel Response

No potential risks beyond the previously discussed issues of recombination, heterologous encapsidation, and synergy were identified. The Panel agreed with the Agency that the benefits to agriculture and horticulture of resistance afforded by PVCP-PIPs needs to be considered along with the possible risks.

Expression of viral proteins that directly contribute to synergy should be avoided. If plants expressing viral proteins contributing to synergy were to be produced and released, they would almost certainly be avoided by producers because of the potential for adverse effects from mixed infection, or infection by the heterologous virus which would have a more deleterious effect due to synergy. The possible risks of synergistic interactions were viewed by the Panel as an agronomic problem for producers, rather than an ecological problem. The only potential ecological risk of synergy envisioned would be the possibility of pollen transmission of the gene conferring synergy to a weedy or wild relative of the crop plant, resulting in an increase of the viral reservoir external to the crop. In the view of the Panel, this would still pose more of an agronomic problem to producers than an ecological risk, unless the wild plants were exposed to viruses not affecting crop plants, but influencing population dynamics of the wild plants. The Panel questioned whether there is any evidence that viruses play a role in controlling populations of wild or weedy plants.

The use of alternative constructs effective through PTGS or other mechanisms – or non-viral mechanisms effective against multiple viruses – is probably favored. RNA-mediated “*resistance*” is frequently superior to resistance observed from PVCP-PIPs, and avoids possible virus-transgene interactions based on CP expression. The pyramiding of genes to confer additional resistances, or multiple mechanisms against a single virus, is also preferred because of the reduced probability of any viral mutant overcoming multiple mechanisms (Hammond et al. 1999).

Cross-protection (pre-inoculation of plants with a mild virus isolate to prevent the deleterious effects of infection by a severe isolate) has been utilized in a number of different crops around the world (Lecoq 1998). There is little difference between PVCP-PIPs and cross-

protection in effect on disease suppression, although PVCP-PIPs generally express lower levels of coat protein than produced by the protective virus used in cross-protection. Cross-protection has been used successfully around the world, with current usage in the US against *Pepino mosaic virus* in tomato. In this case, plants infected early show no symptoms on fruit, whereas plants infected at the time of fruit formation show strong symptoms on the fruit, and fruit may be unsaleable. Another example is papaya production in Hawaii. The use of PRSV CP-transgenic plants in Hawaii is helping to support papaya production, which was in severe decline because of the effects of PRSV. This is an example of a benefit of the PVCP-PIP technology, in a case where no other form of “*resistance*” was available.

REFERENCES

- Aaziz R and Tepfer M (1999). Recombination in RNA viruses and in virus-resistant transgenic plants. *J. Gen. Virol.* 80:1339-1346.
- Allison RF, Schneider WL and Greene AE (1996). Recombination in plants expressing viral transgenes. *Seminars in Virology* 7: 417-422.
- Bejarano ER, Khashoggi A, Witty M and Lichtenstein CP (1996). Integration of multiple repeats of geminiviral DNA into the nuclear genome of tobacco during evolution. *Proc. Natl. Acad. Sci. USA* 93:759-764.
- Cooper JJ and Jones AT (1983). Responses of plants to viruses: Proposals for the use of terms. *Phytopathology* 73:127-128.
- Dardick CD, Taraporewala Z, Lu B and Culver JN (1999). Comparison of tobamovirus coat protein structural features that affect elicitor activity in pepper, eggplant, and tobacco. *Molecular Plant-Microbe Interactions* 12:247-251.
- de Wispelaere M, Gaubert S, Trouilloud S, Belin C and Tepfer M (2004). A map of the diversity of RNA3 recombinants appearing in plants infected with Cucumber mosaic virus and Tomato aspermy virus. *Virology*, in press.
- Dietrich C and Maiss E (2003). Fluorescent labeling reveals spatial separation of potyvirus populations in mixed infected *Nicotiana benthamiana* plants. *J. Gen. Virol.* 84:2871-2876.
- Dinant S, Blaise F, Kusiak C, Astier-Manificier S and Albouy J (1993). Heterologous resistance to the Potato Virus Y in transgenic tobacco plants expressing the coat protein gene of lettuce mosaic potyvirus. *Phytopathology* 83:818-824.
- Ding SW, Shi BJ, Li WX and Symons RH (1996). An interspecific hybrid RNA virus is significantly more virulent than either parental virus. *Proc. Natl. Acad. Sci. USA* 93:7470-7474.
- Duffus JE (1971). The role of weeds in the incidence of virus diseases. *Annual Review of Phytopathology* 9:319-340.
- Estabrook EM, Tsai J and Falk BW (1998). *In vivo* transfer of barley stripe mosaic hordeivirus ribonucleotides to the 5' terminus of maize stripe tenuivirus RNAs. *Proc. Natl. Acad. Sci. USA* 95:8304-8309.
- Farinelli L, Malone P and Collett GF (1992). Heterologous encapsidation of Potato Virus Y strain P (PVY⁰) with the transgenic coat protein of PVY strain N (PVY^N) in *Solanum tuberosum* cv. Bintje. *Bio/Technology* 10:1020-1025.
- Fernández-Cuartero B, Burguán J, Aranda MA, Salánki K, Moriones E and García-Arenal F (1994). Increase in the relative fitness of a plant virus RNA association with its recombinant nature. *Virology* 203:373-377.

Fernandez-Delmond I, Pierrugues O, de Wispelaere M, Guilbaud L, Gaubert S, Divéki Z, Godon C, Tepfer M and Jacquemond M (2004). A novel strategy for creating recombinant infectious RNA virus genomes. *J. Virol. Methods.* 121:247-257.

Fraser RSS and Gerwitz A (1987). In: *Genetics and Plant Pathogenesis*, Day PR and Ellis GJ eds. British Society for Plant Pathology.

Friess N and Maillet J (1997). Influence of cucumber mosaic virus infection on the competitive ability and reproduction of chickweed (*Stellaria media*.). *New Phytologist.*135:667-674.

Fuchs M, Gal-On A, Raccach B, and Gonsalves D (1999). Epidemiology of an aphid nontransmissible potyvirus in fields of nontransgenic and coat protein transgenic squash. *Transgenic Research* 8:429-439.

Gibbs A (1980). A plant virus that partially protects its wild legume host against herbivores. *Intervirology* 13:42-47.

Halfhill MD, Richards HA, Mabon SA and Stewart, Jr. CN (2001). Expression of GFP and Bt transgenes in *Brassica napus* and hybridization with *Brassica rapa*. *Theoretical and Applied Genetics* 103:659–667.

Halfhill MD, Zhu B, Warwick SI, Raymer PL, Millwood RJ, Weissinger AK and Stewart, Jr. CN (2004). Hybridization and backcrossing between transgenic oilseed rape and two related weed species under field conditions. *Environmental Biosafety Research* 3:73-81.

Hammond J, Lecoq H, and Raccach B (1999). Epidemiological risks from mixed virus infections and transgenic plants expressing viral genes. *Advances in Virus Research* 54:189-314.

Hammond J (1982). Plantago as a host of economically important viruses. *Advances in Virus Research* 27:103-140.

Harper G, Osuji O, Heslop-Harrison JS (Pat) and Hull R (1999). Integration of banana streak badnavirus into the Musa genome: molecular and cytological evidence. *Virology* 255:207-213.

Haygood R, Ives AR, and Andow DA. 2004. Population genetics of transgene containment. *Ecology Letters.* 7: 213-220.

Henderson MH and Cooper JI (1977). Effects of thermal shock treatment on symptom expression in test plants inoculated with Potato Aucuba Mosaic Virus. *Annals of Applied Biology.* 86: 389-395.

Hull R (2002). *Matthews' Plant Virology*. Academic Press.

Jones RAC and Nicholas DA (1998). Impact of an insidious virus disease in the legume component on the species balance within self-regenerating annual pasture. *Journal of*

Agricultural Science, Cambridge 131:155-170.

Kyle MM (1993). *Resistance to viral diseases in vegetables: genetics & breeding*. Timber Press.

Lecoq H (1998). Control of Plant Virus Diseases by Cross Protection. In: *Plant Virus Disease Control*, Hadidi A, Khetarpal, RK and Koganezawa H, eds., pp. 33-40. APS Press.

Liu L, Grainger J, Canizares MC, Angell SM and Lomonosoff GP (2004). *Cowpea mosaic virus* RNA-1 acts as an amplicon whose effects can be counteracted by a RNA-2-encoded suppressor of silencing. *Virology* 323:37-48.

Lockhart BE, Menke J, Dahal G and Olszewski NE (2000). Characterization and genomic analysis of tobacco vein clearing virus, a plant pararetrovirus that is transmitted vertically and related to sequences integrated in the host genome. *J. Gen. Virol.* 81:1579-1585.

Maskell LC, Raybould AF, Cooper JI, Edwards ML and Gray AJ (1999). Effects of turnip mosaic virus and turnip yellow mosaic virus on the survival, growth and reproduction of wild cabbage (*Brassica oleracea*). *Annals of Applied Biology* 135:401-407.

Nagy PD, Ogiela C, and Bujarski JJ (1999). Mapping sequences active in homologous RNA recombination in brome mosaic virus: prediction of recombination hot spots. *Virology*. 1999 Feb 1;254(1):92-104.

Nagy PD and Simon AE (1997). New insights into the mechanisms of RNA recombination. *Virology*, 235: 1-9.

Navas ML, Fries N and Maillet J (1998). Influence of cucumber mosaic virus infection on the growth response of *Portulaca oleracea* (purslane) and *Stellaria media* (chickweed) to nitrogen availability. *New Phytologist* 139:301-309.

Potter LR and Jones IT (1981). Interaction between barley yellow dwarf virus and powdery mildew in four barley genotypes. *Plant Pathology*. 330:133-139.

Olsthoorn RC, Bruyere A, Dzianott A, Bujarski JJ. (2002). RNA recombination in brome mosaic virus: effects of strand-specific stem-loop inserts. *J Virol.* 76:12654-62.

Qu F, Ren T, and Morris TJ (2003). The coat protein of *Turnip crinkle virus* suppresses posttranscriptional gene silencing at an early initiation step. *J. Virol.* 77:511-522.

Remold SK (2002). Unapparent virus infection and host fitness in three weedy grass species. *Journal of Ecology* 90:967-977.

Revers F, Le Gall O, Candresse T, Le Romancer M and Dunez J (1996). Frequent occurrence of recombinant potyvirus isolates. *J. Gen. Virol.* 77:1953-1965.

Sacristán S, Fraile A and García-Arenal F (2004). Population dynamics of *Cucumber mosaic*

virus in melon crops and in weeds in Central Spain. *Phytopathology* 94:992-998.

Shapka N and Nagy PD (2004). The AU-rich RNA recombination hot spot sequence of Brome mosaic virus is functional in tombusviruses: Implications for the mechanism of RNA recombination. *J. Virol.* 78, 2288-2300.

Shukla DD, Ward CW, and Brunt AA (1994). *The Potyviridae*. CAB International.

Stark JD and Bank JE (2003). Population-level effects of pesticides and other toxicants on arthropods. *Annual Review of Entomology* 48: 505-519.

Stark JD, Banks JE, Vargas RI (2004). How risky is risk assessment? The Role that Life History Strategies Play in Susceptibility of Species to Stress. *Proc. Natl. Acad. Sci. USA* 101:732-736.

Stewart, Jr. CN, Halfhill MD and Warwick SI (2003). Transgene introgression from genetically modified crops to their wild relatives. *Nature Reviews Genetics* 4:806-817.

Takeshita M, Shigemune N, Kikuhara K, Furuya N and Takanami Y (2004). Spatial analysis for exclusive interactions between subgroups I and II of cucumber mosaic virus in cowpea. *Virology* 328: 45-51.

Tepfer M (2002). Risk assessment of virus resistant transgenic plants. *Annual Review of Phytopathology*. 40: 467-491.

Thomas CL, Leh V, Lederer C and Maule AJ (2003). *Turnip crinkle virus* coat protein mediates suppression of RNA silencing in *Nicotiana benthamiana*. *Virology* 306:33-41.

Truco M, Hu J, Sadowski J and Quiros C (1996). Inter- and intra-genomic homology of the Brassica genomes: implications for their origin and evolution. *Theoretical and Applied Genetics* 93:1225-1233.

Thurston M, Pallett D, Cortina-Borja M, Edwards ML, Raybould AF and Cooper JI (2001). The incidence of viruses in wild Brassica nigra in Dorset (UK). *Annals of Applied Biology* 139, 277-284.

Varrelmann M, and Maiss E (2000). Mutations in the coat protein gene of *Plum pox virus* suppress particle assembly, heterologous encapsidation and complementation in plants of *Nicotiana benthamiana*. *J. Gen. Virol.* 81:567-576.

Walsh JA, Sharpe AG, Jenner CE and Lydiate DJ (1999). Characterisation of resistance to turnip mosaic virus in oilseed rape (*Brassica napus*) and genetic mapping of *TuRB01*. *Theoretical and Applied Genetics* 99:1149-1154.

Wierzoslawski R, Dzianott A, Bujarski J. (2004). Dissecting the requirement for subgenomic promoter sequences by RNA recombination of brome mosaic virus in vivo: evidence for functional separation of transcription and recombination. *J Virol.* 78:8552-64.

Zhu B, Lawrence JR, Warwick S, Mason P, Braun L, Halfhill M and Stewart, Jr. CN (2004). Inheritance of GFP-Bt transgenes from *Brassica napus* in backcrosses with three wild *B. rapa* accessions. *Environmental Biosafety Research* 3:45-54.