UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES, AND
TOXIC SUBSTANCES

March 1, 2006

MEMORANDUM

SUBJECT: Transmittal of Revised Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting Held December 6-8, 2005 on Plant-Incorporated Protectants Based on Virus Coat Protein Genes: Science Issues Associated with the Proposed Rule

TO: Clifford J. Gabriel, Ph.D.
Director
Office of Science Coordination and Policy

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

Joseph E. Bailey
Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Attached, please find revised meeting minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, Virginia on December 6-8, 2005. This report addresses a set of scientific issues being considered by the Environmental Protection Agency pertaining to Plant-Incorporated Protectants Based on Virus Coat Protein Genes: Science Issues Associated with the Proposed Rule.

Subsequent to issuance of the final minutes, one of the Panelists from this meeting, Dr. Shou-Wei Ding, noted an error and provided a clarification. The minutes have been revised as follows:

Page 12, sentence reading:

If designed for post-translational gene silencing (PTGS), this restriction need not apply.

has been changed to:

If designed for post-transcriptional gene silencing (PTGS), this restriction need not apply.

Page 13, section title reading:

Post-Translational Gene Silencing

has been changed to:

Post-Transcriptional Gene Silencing

Sentence reading:

Post-translational gene silencing (PTGS) is a highly desirable transgenic resistance strategy for virus resistance because 1) it is based on a natural plant defense mechanism against viruses themselves, 2) transgenes can easily be designed to produce only RNA, not protein, and 3) PTGS gives stronger resistance than protein methods.

has been changed to:

PTGS is a highly desirable transgenic resistance strategy for virus resistance because 1) it is based on a natural plant defense mechanism against viruses themselves, 2) transgenes can easily be designed to produce only RNA, not protein, and 3) PTGS gives stronger resistance than protein methods.

Sentence reading:

Two types of PTGS-inducing transgenes can be shown to express no protein: 1) transgene insertions, and 2) insertions of transcribed inverted repeat (IR) constructs.

has been changed to:

Two types of PTGS-inducing transgenes can be shown to express no protein: 1) transgene insertions that cannot be translated into proteins, and 2) insertions of transcribed inverted repeat (IR) constructs.

Page 26, sentence reading:

If the sequence was designed for post-translational gene silencing (PTGS), this restriction does not apply.

has been changed to:

If the sequence was designed for PTGS, this restriction does not apply.

Attachment

cc:

Susan Hazen
Margaret Schneider
Amy Farrell
Jim Jones
Anne Lindsay
Margie Fehrenbach
Janet Andersen
Debbie Edwards
Steven Bradbury
William Diamond
Arnold Layne
Tina Levine
Lois Rossi
Frank Sanders
Randolph Perfetti
William Jordan
Douglas Parsons
Enesta Jones
Vanessa Vu (SAB)
Tom McClintock
Elizabeth Milewski
Anne Fairbrother
Melissa Kramer
Rebecca Edlestein
Tessa Milofsky
OPP Docket

FIFRA Scientific Advisory Panel Members

Stephen M. Roberts, Ph.D. (Chair of the FIFRA SAP)
John Bucher, Ph.D.
Janice E. Chambers, Ph.D.
H. Christopher Frey, Ph.D.
Stuart Handwerger, M.D.
Steven G. Heeringa, Ph.D.
Gary Isom, Ph.D.
Kenneth M. Portier, Ph.D.

FQPA Science Review Board Members

Judith Bender, Ph.D.
Judith Brown, Ph.D.
George Cobb, Ph.D.
Shou Wei Ding, Ph.D.
Bryce W. Falk, Ph.D.
Steven Gendel, Ph.D.
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Steven A. Lommel, Ph.D.
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Geoffrey I. Scott, Ph.D.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES, AND
TOXIC SUBSTANCES

February 28, 2006

MEMORANDUM

SUBJECT: Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting Held December 6-8, 2005 on Plant-Incorporated Protectants Based on Virus Coat Protein Genes: Science Issues Associated with the Proposed Rule

TO: Clifford J. Gabriel, Ph.D.
Director
Office of Science Coordination and Policy

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

Joseph E. Bailey
Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

THRU: Steven Knott, Acting Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Attached, please find the meeting minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, Virginia on December 6-8, 2005. This report addresses a set of scientific issues being considered by the Environmental Protection Agency pertaining to Plant-Incorporated Protectants Based on Virus Coat Protein Genes: Science Issues Associated with the Proposed Rule.

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SAP Report No. 2006-01

MEETING MINUTES

FIFRA Scientific Advisory Panel Meeting, December 6-8, 2005, held at the Holiday Inn - National Airport Hotel in Arlington, VA 22202

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

PLANT-INCORPORATED PROTECTANTS BASED ON VIRUS COAT PROTEIN GENES: SCIENCE ISSUES ASSOCIATED WITH THE PROPOSED RULE

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/, http://www.regulations.gov 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.

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

PLANT-INCORPORATED PROTECTANTS BASED ON VIRUS COAT PROTEIN GENES: SCIENCE ISSUES ASSOCIATED WITH THE PROPOSED RULE

Stephen M. Roberts, Ph.D.
FIFRA SAP Session Chair
FIFRA Scientific Advisory Panel
Date: February 28, 2006

Steven G. Heeringa, Ph.D.
FIFRA SAP Chair
FIFRA Scientific Advisory Panel
Date: February 28, 2006

Paul I. Lewis, Ph.D.
Designated Federal Official
FIFRA Scientific Advisory Panel
Date: February 28, 2006

Federal Insecticide, Fungicide, and Rodenticide Act
Scientific Advisory Panel Meeting
December 6-8, 2005

PLANT-INCORPORATED PROTECTANTS BASED ON VIRUS COAT PROTEIN GENES: SCIENCE ISSUES ASSOCIATED WITH THE PROPOSED RULE

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

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Stuart Handwerger, M.D., Professor of Pediatrics, Professor of Cell Biology, University of Cincinnati, Children's Hospital Medical Center, Cincinnati, OH

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

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Shou Wei Ding, Ph.D., Professor, Department of Plant Pathology, University of California at Riverside, Riverside, CA

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Lars K. Poulsen, Ph.D., M.D., Professor and Head of Research, Allergy Clinic, National University Hospital, Copenhagen, Denmark

Geoffrey I. Scott, Ph.D., Director, U.S. Department of Commerce, NOAA, National Ocean Service, Center for Coastal Environmental Health & Biomolecular Research at Charleston, Charleston, SC

PUBLIC COMMENTERS

Oral statements were made by:

Keith Redenbaugh, Ph.D., Seminis Vegetable Seeds
Michael Watson, Ph.D., United States Department of Agriculture
James White, Ph.D., United States Department of Agriculture

Written statements were provided by:
Roger Beachy, Ph.D., Danforth Center
Myron Brakke, Ph.D., University of Nebraska-Lincoln
Stella Coakley, Ph.D., The American Phytopathological Society
R. James Cook, University of Washington
Alison Power, Ph.D., Cornell University
Hector Quemada, Ph.D.,Western Michigan University
Keith Reding, Ph.D., Monsanto Company
Keith Redenbaugh, Ph.D., Seminis Vegetable Seeds
Luis Sequeira, Ph.D., University of Wisconsin

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 Environmental Protection Agency pertaining to its review of plant-incorporated protectants based on virus coat protein genes: science issues associated with the proposed rule. Advance notice of the meeting was published in the *Federal Register* on September 23, 2005. The review was conducted in an open Panel meeting held in Arlington, Virginia, from December 6-8, 2005. The meeting was chaired by Stephen Roberts, Ph.D. Paul Lewis, Ph.D., served as the Designated Federal Official. J. Thomas McClintock, Ph.D. (Director, Hazard Assessment Coordination and Policy Division, Office of Science Coordination and Policy, EPA), welcomed the Panel to the meeting. Janet Andersen, Ph.D. (Director, Biopesticides and Pollution Prevention Division, Office of Pesticide Programs, EPA), provided opening remarks. Sharlene Matten, Ph.D. (Office of Pesticide Programs, EPA), offered a regulatory overview of PIPs. Elizabeth Milewski, Ph.D. (Office of Science Coordination and Policy, EPA), followed with a discussion on PVCP-PIPs. Anne Fairbrother, Ph.D. (National Health and Environmental Effects Research Laboratory, ORD, EPA), provided a summary of EPA's position on gene flow and its environmental impact. Melissa Kramer (Office of Science Coordination and Policy, EPA), presented a summary of viral interactions and post-transcriptional gene silencing. Rebecca Edelstein, Ph.D. (Office of Pesticide Programs, EPA), gave a summary of EPA's position on issues associated with exposure to PVC-proteins. Tessa Milofsky, M.S. (Office of Pesticide Programs, EPA), ended the session with a summary of EPA's position on environmental safety of selectable markers.

SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

Gene Flow and Proposed Exemption

The Panel was supportive of the Agency's intent to exempt from regulation any PVCP-PIP crops that (1) do not have sexually compatible wild relatives in the location of intended cultivation (US & Territories), and (2) are not likely to become weedy themselves. Some crop plants known to be capable of becoming weedy and/or of hybridizing with a crop species to produce a wild hybrid were, however, included on the Agency's proposed list. To address this issue, the Panel recommended a set of criteria to evaluate species to help determine whether they should be included or excluded from the list of PVCP-PIP-containing crops to be exempted from EPA regulation. Consultation with taxon-specific biological experts was recommended in order to collate specific information regarding the potential for outward gene flow to a relative and/or of weediness potential of a candidate crop species if hybridization became a reality. Consultation was recommended because this information is likely to be primarily unavailable or inaccessible as published literature. It was suggested that if sufficient geographic or specific ecological barriers to pollen flow could be demonstrated, exemption could be granted, according to advice from specialists on that crop species and its relatives. In summary, the Panel recommended that all PVCP-PIP crops on the proposed exempt list and any additional crops to be proposed for consideration of exemption should meet proposed criteria prior to being granted exemption from regulation.

The 2004 FIFRA SAP reviewing PVCP-PIPs concluded that PVCP-PIPs would not have a negative environmental effect and that "it would be unlikely that a plant population freed from viral pressure would give a plant species a competitive advantage." Members of the current Panel had differing opinions about potential effects PVCP-PIPs may have on plant population dynamics and some opinions differed slightly from both the Agency and the 2004 SAP. Some concluded that given a limited body of more recent research, as well as EPA indicating a lack of data on this topic, concluding that viruses typically have no effect on their wild plant hosts is not accurate. Other members of the current Panel continued to agree with the previous SAP Panel. Because of the differing opinions, the Panel agreed that additional research is necessary to provide stronger support for the two current positions. Employment of multiple, highly effective biological containment and/or mitigation methods could provide one means of releasing the PVCP-PIP crop with sexually compatible wild relatives. An important ecological risk associated with gene flow from crop plants into wild relatives is that acquisition of crop genes could alter wild plant population dynamics either by allowing the wild species to persist in larger populations across a larger geographic range, or in a wider range of habitats, e.g., collectively, “increased weediness”. Although the Panel recommended no general exemption for PVCP-PIP crops with sexually compatible wild relatives, it suggested that ecological risk assessment tools should be considered that would evaluate fitness effects, or population-level consequences, associated with PVCP-PIPs introgressed into wild populations. If the PVCP-PIP is found to increase wild plant fitness, the next step in a risk assessment would be matrix modeling. Such studies are time consuming, but important. Simply surveying populations of the wild plant relatives for virus infection is unlikely, in itself, to provide meaningful data. Since the Panel

recommended that there be no general exemption for PVCP-PIP crops with sexually compatible wild relatives, the Panel did not discuss additional methods to determine weedy, invasive, or threatened/endangered status of those relatives.

Novel Viral Interactions and Recombination

The Panel agreed that the likelihood for "novel interactions" is very low, and the environmental concerns that might result from potential novel viral interactions that could result from the use of PVCP-PIPs is lower than that which occurs naturally from mixed virus infections. The general consensus was that if a viral sequence was engineered to express a protein, the sequence should be from a virus originating in the United States, its possessions or territories. If designed for post-transcriptional gene silencing (PTGS), this restriction need not apply.

Three biological entities influence recombination; the plant host, the virus from which the PVCP is derived, and super-infecting viruses. The Panel envisioned several scenarios of potential recombination in which a complete virus coat protein (CP) [or other viral protein] would serve as the PVCP. It was agreed that recombination rarely results in an incrementally higher probability of a virus arising with new, and possibly undesirable properties. A related discussion centered on the likelihood, frequency, and consequences of virus recombination between the PIP and a virus infecting the PIP plant. It was concluded that the shorter the PIP sequence, the less likely recombination would occur. One view was that recombination frequency was irrelevant, and that instead, the important outcome was the consequence of the recombination. PTGS results in the production of small RNAs from the PIP sequence, and so an infecting virus could, in certain circumstances, become recombinatorial. While the discussion was wide-ranging, the consequences of any viable recombination event were considered to be minimal. Most currently deployed PVCP-PIPs with virus resistance involve PTGS instead of CP expression, and PTGS does not require the expression of CP or other viral proteins. The Panel recommended exempting any PIP designed to induce PTGS that does not encode a viral protein and developed a decision flow-chart incorporating criteria for exemptions to enable rapid and effective decision-making regarding risk. When a PIP expresses a CP, the transgene should be subjected to the decision flow chart to take into account the relative likelihood and consequence of recombination between the PIP and a super-infecting virus. The decision flow-chart determines those potential situations, which if determined undesirable, would warrant further review.

Nontarget and Human Non-dietary Exposure - Allergenicity

Viral coat proteins are naturally present in the environment and no adverse effects to humans or non-targets have been reported. Initially, this leads to the expectation that risks will be minimal. A correct position would be to proceed with care because genes transfected into plants have the potential to persist in the environment. Even though no plant virus coat protein has been identified as an allergen, and further that VCP-PIPs are natural sequences and structures, the probability of expression in all plant parts (*specifically in pollens)* may be problematic. Pollens are major sources of inhaled allergens. The Panel agreed that dermal exposure to PVCP-PIPs poses far lower risk than inhalation exposure. Based on the allergenic potential of PVCP-PIPS, the Panel concluded that the factors presented by the Agency are useful,

but incomplete to allow regulatory exemption of PVCP-PIPs. The Panel proposed a decision tree that adds to the proposed factors and can be merged with considerations related to gene flow and recombination discussed above.

Post-transcriptional Gene Silencing

PTGS is a highly desirable transgenic resistance strategy for virus resistance because 1) it is based on a natural plant defense mechanism against viruses themselves, 2) transgenes can easily be designed to produce only RNA, not protein, and 3) PTGS gives stronger resistance than protein methods. This is the premier virus resistance strategy for use today and into the future. Two types of PTGS-inducing transgenes can be shown to express no protein: 1) transgene insertions that cannot be translated into proteins, and 2) insertions of transcribed inverted repeat (IR) constructs. Whether a transgenic plant falls into the IR transgene category must be determined by the nature of the transgene insertion structure in the plant genome, not the original design. Based on current information, a PTGS transgene has the same likelihood of gene flow into new plant species as a protein-mediated virus resistance transgene. As well, in terms of conferring a potential advantage to a wild or weedy relative, PTGS and protein-mediated virus resistance transgenes should have the same impact unless one transgene confers better resistance. Recombination between a full-length viral RNA and a cleaved small RNA resulting from PTGS would yield a truncated non-functional RNA and so PTGS transgenes pose negligible potential to yield recombinant viruses.

Post-translational Modifications

Historically, virus infected plants have been a part of the human and domestic animal food supply without adverse human or animal health effects. Exposure to PVC proteins generated in PVCP-PIPs that are identical with or within the range of natural variation of plant virus coat proteins would not alter the risk of toxicity and allergenicity. Thus, prior knowledge of natural variation limits of the individual PVC proteins is required. There are no general principles that can be used to determine how relative risk increases with an increasing number of added amino acids. The addition of other amino acids containing reactive side chains that can serve as sites for post-translational modifications may be significant and should be evaluated on a case-by-case basis. It is difficult to identify modifications that would be expected to be "within the range of natural variation for all virus families". This would require prior knowledge of the natural variation limits of the individual PVC proteins, which is not available. Modifications such as single amino acid substitutions with biochemically similar amino acids that do not affect secondary or tertiary structure might be of relatively little concern. Given the possible range of natural variations for PVC proteins, it would be appropriate to assess whether specific modifications are within natural variation limits of the PVC protein case-by-case.

Regarding food safety of PVC-proteins and post-translational modifications, the Panel agreed that it appears appropriate to use naturally occurring PVC-proteins that are currently consumed in foods, and any available information on naturally occurring variants, as comparators for the minimally modified proteins. Given the large number of potential modifications that might be made in a PVC-protein, it appears to be appropriate to evaluate each modification on a case-by-case basis. This includes both the determination that the protein is in

fact minimally modified and whether the modified protein is as safe as an unmodified protein. However, the Panel was not able to identify a clear set of criteria that can be used to define minimally modified. The Panel recognized the possibility that any modifications that are sufficient to move the protein out of "minimally modified" category would reduce or eliminate the efficacy of the construct. Further, to the extent that data are available, it is appropriate to compare modified PVC-proteins to the range of existing natural variants. Similarly, constructs that produce no protein describe a situation with minimal food safety risk. It might be appropriate to consider distinguishing situations in which the construct can never produce protein, from one where no protein is produced from a functional gene construct.

Chimeric PVC-Proteins

The Panel was unclear on the stated definition of 'a chimeric protein...[given as] the fusion between two or more capsid proteins', or as interpreted by the Panel, a completely novel protein that differed from a minimally modified one. The Panel indicated that it may be difficult to make specific predictions regarding toxicity and/or allergenicity relative to wild type CP molecules in a chimeric protein. Expression levels may be important but the capacity to form multimers or aggregates, which differ from those formed by the original CPs, is an undesirable feature. Specific sequences of the protein should be taken into account separately, and include: the N-terminal methionine and signal/leader sequences, targeting signals, ER-retention signals, and tagging sequences.

Selectable Marker Genes

Selectable marker genes are incorporated into constructs and inserted into plants to select those plants that have been successfully transformed. They fall into three groups: herbicide resistance, antibiotic resistance, and other. The antibiotic resistance marker (NPTII) and others (GUS, PMI) should be exempt in plant species determined to be 'low risk', based on previously discussed criteria. The herbicide markers (CP4 EPSPS, GOX/GOXv247, PAT) should not be exempted. They should be considered on a case-by-case basis taking into account ferality potential, because glyphosate and glufosinate are commonly used to eradicate feral species from non-agricultural ecosystems, and volunteers within agroecosystems. These markers would preclude the use of the respective herbicides. Because selectable marker genes are expressed, they should be considered together with the PVCP-PIP. Three environments are of concern: agronomic environments [arable, pasture, forest, fish farms, having continuous inputs], peri-agricultural environments which comprise the edges of agronomic environments where some pesticides are applied or received from agronomic environments, and natural environments with no pesticide use. Although interactions occur among the three environments, this question was considered most relevant to natural environments. Selectable markers and PVCP-PIPs may pose variable risks. Thus, monitoring gene flow, risk containment, and risk mitigation is needed case-by-case, with attention on safety and risks to crops, and evaluation of risk mitigation/containment mechanisms.

Criteria and Applicability for Other PIPs

Other PIPs conferring virus resistance should be evaluated similarly as are the PVCP-PIPs, if the PIP's mode of action is via PTGS. There is already extensive experience with PVCP-PIPs, particularly in squash and papayas, and the use of other types of resistance genes without detectable novel or negative effects, which should be considered in future exemption considerations. It is important to build from the positive effects resulting from using those genes in U.S. and worldwide agriculture, and not only the potential negative effects. The Panel mentioned several phenotypes that are associated with specific virus proteins and suggested that perhaps they should not be exempt if protein is expressed from the construct. Knowledge of previous exposures and the nature of natural variation serve as the appropriate comparators for any risk assessment. Finally, the Panel specifically commented on the Agency's background document and the 1992 FIFRA SAP review of PVCP-PIPs. In those documents/reviews, the statement that expression levels of genes other than CP in transgenic plants are fundamentally different than for CPs is incorrect. Suggesting that CPs are the most highly viral expressed proteins, and that expressing other than virus CPs will result in higher levels than are found naturally in virus infections and so may increase potential for interactions is not a valid assumption. The *Potyviridae* (picornavirus lineage) express all gene products equally. The 1992 Panel discussion of protein turnover may not have reached accurate conclusions. For potyviruses, many of the gene products do not turn over – instead they accumulate as distinct inclusion bodies. On a per cell basis, it is almost certain that all viral gene products are expressed at higher levels in virus-infected than transgenic plants. If frequency of product per unit of space and time affects probability for interaction, then probability for interaction is likely to be greater in a virus-infected plant than in a transgenic plant. The Panel acknowledges the the state-of-the-science of plant virology has advanced considerably from 1992 to present, thus contributing to differing conclusions in 1992 and today.

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.

Question 1 - Proposed List of Exempt Plant Species and Selective Advantage

Panel Summary

The Panel was supportive of the intent of the Agency to exempt from regulation those PVCP-PIP crops that 1) do not have sexually compatible wild relatives in the US, and 2) are not likely to become weedy themselves. However, the proposed list of crops clearly includes some crops that are known to form weedy populations and/or produce crop-wild hybrids. For this reason, the Panel recommended implementing a set of criteria that must be met before including any PVCP-PIP-containing crops in a list of species that would be exempt from regulation by the Agency. When determining whether crops meet the criteria for inclusion in the list of exempt species, the Panel strongly recommended consulting with taxon-specific experts due to the fact that, without detailed knowledge of the biology of the crop and its wild relatives, it is not possible to determine if the crop either 1) has the potential to become feral or weedy, or 2)

produce viable crop-wild hybrids. Although breeders, agronomists, and/or ecologists working on particular groups may have this information, it is typically difficult to find (or is not present in) the published literature

Question 1 (a)

Does this list identify plant species that would present low risk of conferring any selective advantage on a wild or weedy relative in the United States, its possessions, or territories were they to contain a PVCP-PIP? Please explain the basis for your answer, providing documentation to support your decision.

Panel Response

Based on the advice of agronomists and ecologists working with some of the included crops, it is clear that the list contains some species that form viable crop-wild hybrids. For example, barley can hybridize with *Hordeum jubatum,* which is a weed in the USA (public comment by Alison Power, Cornell University). Many of the *Prunus* species can cross with each other (including apricot with almond, which are included on the Agency's list), and watermelon crosses with citron melon (*Citrullus lanatus* var. *citroides*; public comment provided by Hector Quemada, Western Michigan University). There may be other crops on the current Agency list that also form viable crop-wild hybrids. The Panel recognized that determining whether a particular crop forms viable hybrids with wild relatives is difficult to ascertain from the literature. For this reason, the Panel recommended consulting agronomists, breeders, and/or ecologists with specialized expertise before including any crop on a list of exempt species (also see response to Question 1(c) below). While the Panel differed in its views on whether to recommend against granting exemptions to any species with sexually compatible wild relatives in the US or its possessions (see Question 2), one Panel member believed that if there are sufficient geographic or other ecological barriers to pollen flow, an exemption could be given based on the advice of experts in the field who are knowledgeable about the given crop and its relatives.

Question 1(b)

What data supports or refutes the rationale above that naturalized populations of plants on the list in question 1(a) would not be expected to become weedy or invasive outside of agricultural fields if they were to acquire virus resistance from a PVCP-PIP (assuming that the cultivated crop is negatively affected by virus infection and a PVCP-PIP targeted at that virus is developed)? If the rationale does not apply to all the crops on this list, is there an alternative rationale that would apply to particular plant species?

Panel Response

As noted in Appendix I of the Agency's background document "Draft Approach to Exempting Certain PVCP-PIPs from Regulation under FIFRA" many crops, including many on the proposed exempt list, form naturalized populations. The Panel noted that there have been many species that are naturalized, but have not spread from the original focus in over a century

(e.g., irises, rhubarb, callas, and Easter lilies planted near farmhouses, long abandoned, have naturalized where planted, but have demonstrated no weedy potential) and saw no reason to expect they will spread in the future. Determining whether a particular crop can naturalize and then spread as a weedy species is difficult to ascertain from the literature and determining the probability that a crop will be more weedy or invasive if it contains a PVCP-PIP is even more difficult. Although the Agency asserts in Appendix I that acquisition of a single trait, such as virus resistance, is not likely to "provide sufficient competitive advantage to make naturalized populations of these plants significant weed problems outside of agricultural fields," no evidence is presented in support of this point of view. The Panel noted that PVCP-PIP crops are developed when virus infection of a crop reduces the crop yield, suggesting that virus infection is quite likely in naturalized populations of the crop as well. The Panel recommended consulting agronomists, breeders, and/or ecologists with specialized (taxon-specific) expertise on weedy populations before including any crop on a list of exempt species. The Panel also recommended against granting exemptions to any crop species that currently form invasive or weedy populations (see Question 1(c)).

Advice of agronomists and ecologists working with some of the included crops indicated that the list contains some species that form weedy or naturalized populations. Examples include the following: 1) *Prunus serotina* (black cherry), a major weed in Europe (the Panel did not have expertise with the plant in the United States) and 2) guava, an important feral tree that can displace other species in "natural" Hawaiian ecosystems. It has been reported that two guava species introduced in the 1880s (*Psidium guajava* and *Psidium catteianum) are,* both are serious pasture weeds and the second is a very serious weed in native forest ecosystems, and are disseminated by birds and wild pigs. The latter is also a weed in south Florida (personal communication) and 3) olives which are a major feral weedy tree in Europe, Africa (Breton et al., 2005), New-Zealand, and Australia (Spennemann, and Allen, 2000), especially where cultivation is abandoned. Others have described feral olive infestations in the Channel Islands National Park, and in oak woodlands and forest in Sonoma Valley and Davis, CA. In California, olive is "considered an invasive exotic" that "compete[s] with native flora" (personal communications)

Question 1(c)

Please list any additional plants (including genus and species) that both: (1) have no wild or weedy relatives in the United States, its possessions, or territories with which they can form viable hybrids in nature and (2) have low potential to naturalize and become weedy or invasive outside of agricultural fields with the acquisition of any PVCP-PIP. For each identified plant please explain why (2) is likely the case.

Panel Response

It is very difficult to identify crops that have no sexually compatible wild or weedy relatives in the US or its possessions and that do not become weedy or invasive themselves. This information is unique to each crop, is often not published, and is often known only by the agronomists, breeders, and ecologists working with the specific taxa in question. Similarly, it is hard to predict which introduced ornamental tree species will become feral (Kowarik 2005), and

under what circumstances a wild native species might become an invasive weed (e.g., *Amaranthus rudus* in the Midwest). It is easier to make such predictions for highly domesticated crops. For these reasons, the Panel recommended that all PVCP-PIP crops (i.e., crops on the proposed exempt list and any additional crops) meet the following set of criteria proposed by the Panel before an exemption from regulation is granted.

1. A crop should be included on the exempt list if it forms no viable hybrids with wild or weedy relatives anywhere in the US or its possessions. The inability to form hybrids must be verified by breeders, agronomists and/or others with experience with the genus.

Panel members held differing opinions about the geographic and topographic separation necessary to prevent gene flow. One Panel member believed that crops with sexually compatible wild relatives in sufficiently distant ecosystems do not pose a risk; another concluded that sexually compatible wild relatives anywhere in the continental US (for crops that will be planted in the continental US) should preclude exemption. No panel member thought that a species should be excluded from a list for the continental US if it posed a risk in a far removed US possession (e.g., Guam).

2. A crop should not be included on the exempt list only if it is currently weedy or invasive. Current status as not weedy or invasive should be verified by breeders, agronomists, and ecologists familiar with the crop. The Agency should not rely solely on currently available online sources (even if these sources are governmental) when determining current weediness.

3. A crop should be included on the exempt list if, in the judgment of breeders, agronomists, and ecologists familiar with the species, it will not establish weedy or invasive populations if it becomes virus resistant (due to a PVCP-PIP). Experimental evidence that virus resistance will not significantly increase the competitiveness of naturalized plants should be provided if the consensus of the experts deem this to be necessary. Such evidence could be obtained from studies in which virus-resistant (transgenic) and virus-susceptible (non-transgenic) cultivars are compared in a weedy environment in the presence of natural virus infection. Also, a competition experiment, such as that referenced by Sukopp et al. (2005) between sugarbeet and weedy beet, would be adequate to provide such data.

4. If a PVCP-PIP crop has the potential to naturalize, but the PVCP-PIP transgene is in biocontainment and/or in biomitigation constructs that are stacked such that escapes from cultivation are too unfit to compete with the wild type, a consensus of breeders, agronomists, and ecologists, or others with experience with the species could advise addition to the list.

The Panel emphasized that all crop species should be subjected to the criteria listed above before being included on the Agency's exempt list. However, without consulting relevant experts or doing an extensive literature review, the Panel suggested (based on Ellstrand 2003) that cotton (*Gossypium hirsutum* and *G. barbadense*), alfalfa (*Medicago sativa*), peanut (*Arachis hypogea*), pearl millet (*Pennisetum glaucum*), and many ornamentals may well meet the criteria listed above.

Question 2 - Criteria to Assess Gene Flow and Environmental Concerns

Panel Summary

The Panel recommended against granting general exemptions to any crops having sexually compatible wild relatives, regardless of whether (or not) those relatives are weedy, invasive, or threatened/endangered. (Sexually compatible refers to the possibility of having crop transgenes backcross and introgress into the relative; it does not refer to sterile hybrids.) The Panel suggested that if multiple, highly effective biological containment and/or biological mitigation methods were implemented, it would be possible to release PVCP-PIP crops with sexually compatible wild relatives. Because the Panel differed in its opinions about the effects PCVP-PIPs may have on plant population dynamics, they cautioned that further research is needed to provide stronger support for this particular issue.

Question 2(a)

Please comment on whether the following criteria would allow the Agency to identify correctly those PVCP-PIPs that present low risk with respect to environmental concerns associated with gene flow of a PVCP-PIP. What data supports or refutes the Agency's rationale for developing these criteria?
(i)the plant containing the PVCP-PIP is itself not a weedy or invasive species outside of agricultural fields in the United States, its possessions, or territories, *and*
(ii)the plant containing the PVCP-PIP does not have relatives outside of agricultural fields in the United States, its possessions, or territories that are weedy or invasive species or endangered/threatened species with which it can produce viable hybrids in nature.

Panel Response

Because the Panel did not recommend a general exemption for PVCP-PIP crops with sexually compatible wild relatives, the Panel did not discuss specific criteria (2(a)i and 2(a)ii) in any detail. An important ecological risk associated with gene flow from crop plants into their wild relatives is that the acquisition of crop genes might substantially alter the population dynamics of the wild plant. In particular, a transgene introgressed from the crop relative into a wild population might allow the wild species to persist in larger populations across a larger geographic range, or in a wider range of habitats. Collectively these changes in population dynamics can be considered "increased weediness".

The probability that a particular transgene will lead to increased weediness depends on the phenotype conferred by the transgene and on the ecological factor(s) currently limiting the size or distribution of the wild species. In particular, if the transgene alters plant response to the ecological factor limiting population size, then population dynamics may be affected. For PVCP-PIPs, the relevant consideration is whether virus resistance (conferred by the PVCP-PIP) leads to changes in the size or distribution of wild plant species with the PVCP-PIP. By contrast, the probability that a particular transgene alters the dynamics of a wild relative cannot be predicted by the current status of the wild species as weedy, invasive, or threatened/endangered.

The Panel agreed that the criteria proposed by the Agency would not correctly identify PVCP-PIPs which pose unacceptable environmental risks.

Question 2(b)

Are there other factors besides a plant's weediness, invasiveness, and/or endangered/threatened status that should be taken into consideration when evaluating whether a PVCP-PIP poses low risk with respect to environmental concerns associated with gene flow of a PVCP-PIP?

Panel Response

Although the Panel recommended no general exemption for PVCP-PIP crops with sexually compatible wild relatives, it is possible to evaluate risk on a case-by-case basis. A PVCP-PIP will not have large effects on the wild population unless it increases to high frequency by natural selection. Thus, the first step in an ecological risk assessment should be to evaluate the fitness effects of the PVCP-PIP introgressed into the wild population (e.g., Snow et al. 2003; Fuchs et al. 2004). If the PVCP-PIP has no effect on wild plant fitness, then natural selection will not act to increase its frequency, and it probably poses low risk to the environment. Conversely, if the PVCP-PIP increases wild plant fitness, there is the potential for increased weediness. Thus, the Panel was concerned about PVCP-PIP crops that increase wild plant fitness and emphasized the need for additional research in this area.

If a PVCP-PIP increases the fitness of wild plants, the next step in a complete ecological risk assessment would be to evaluate the population-level consequences of the PVCP-PIP. The potential for increased weediness is difficult to evaluate because little is known about factors controlling population size in plant populations in general, including those that are currently stable, as well as those that are currently weedy or invasive. Several authors advocate the use of population matrix models to evaluate ecological risks associated with the movement of transgenes into wild populations (Parker and Kareiva 1996; Bullock 1999; Hails and Morley 2005). Matrix modeling methods are developed in Caswell (2001) and widely applied in ecology and conservation biology. This approach involves gathering demographic data (probabilities of survival and fecundity through the life cycle) and using this information to project population growth (lambda) into the future. The advantage of this approach is that the relative importance of each demographic transition (i.e., the probability of surviving from each age or life-stage to the next and age-specific fecundities) to population growth can be determined using elasticity analysis (Caswell 2001). The strategy when evaluating the potential consequences of a transgene would be to first use these methods to estimate lambda and elasticities in a non-transgenic population, and then to determine what demographic transition(s) is (are) most affected by virus infection. For example, virus infection might typically kill seedlings; thus, transgenic resistance might lead to an increase in seedling survival. Evaluating the potential ecological effects of this transgene then depends on the importance of the seedling survival to population growth (i.e. the elasticity of seedling survival). For example, if seedling survival is relatively unimportant for population growth (perhaps because density-dependence causes the number of survivors to remain relatively constant) then transgenic virus resistance is likely to have little effect on population growth. Conversely, seedling survival might have large

effects on population growth (perhaps because an increase in seedling survivorship leads to an increase in the number of reproductive plants). In this case transgenic virus resistance could lead to a significant increase in population growth, and perhaps increased weediness.

If a population matrix model suggests that a transgene affects a demographic transition that is currently limiting population size, then it is likely that acquisition of that transgene would lead to changes in population size, if indeed the assumptions used in the model can be validated. One member cautioned that population models can lead to erroneous conclusions (including the member's own model) and thus unvalidated models cannot be used as proof, either way (Gressel, 2005). For example, if a wild population is currently limited by virus infection (perhaps because infected seedlings die before reaching reproductive maturity), then acquisition of a PVCP-PIP could lead to larger populations (by increasing the probability of seedling survival). If, instead, the wild population is limited by an ecological factor unrelated to virus infection (such as safe sites for germination, which places an upper limit on the number of seedlings) then acquiring a PVCP-PIP would have no effect on population dynamics (e.g., seedling survival might increase, more seeds might be produced, but these additional seeds would not result in more plants in the next generation).

These sorts of ecological studies are very time consuming, but very important. It is clear that more research is needed in order to better understand viruses and virus resistance effects in natural/wild plant populations. Simply surveying populations of the wild plant relatives for virus infection is unlikely, in itself, to provide meaningful data. As was clearly noted by the current SAP, virus incidence does not equate with virus pathogenicity (or virus disease) (Bawden, 1964; Hull, 2001). Not all viruses have pathogenic effects (Dawson, 1999), and in fact, virus disease often results only when new plants or new viruses are introduced to a new location (Dawson and Hilf, 1992; Dawson, 1999). Some Panelists also pointed to the fact that natural resistance genes to control viral, bacterial and fungal diseases of crops have been widely used over a long period of time with no evidence of any significant environmental impact among wild plant relatives, and believed that there would most likely be no differences in environmental impact between these natural genes and those (PVCP-PIPs) introduced by genetic manipulation technology. Other Panelists pointed out that conventionally bred resistance genes are likely to be present in wild populations already, and thus, no novel genes have moved into wild populations.

As noted above for PVCP-PIPs, the relevant consideration is whether virus resistance (conferred by the PVCP-PIP) leads to changes in the size or distribution of wild plant species with the PVCP-PIP. However, there are few studies evaluating the effects of infection (or resistance) on individual fitness, population dynamics, and plant community structure in wild plant populations.

Some Panelists believe that virus evolution leads to a balance with the host in natural populations where the virus does not act as a pathogen (i.e., that natural selection always favors decreased virulence). The implication of this belief is that viruses will so rarely have negative effects on host fitness that concerns about PVCP-PIPs in wild populations are unfounded. However, as pointed out by another panelist, there is a very well developed and empirically supported body of theory dealing with the evolution of virulence (the detrimental effects of viruses, pathogens, and parasites on their hosts) (Futuyma 1998; Freeman and Herron 2001;

May and Anderson 1983; Anderson and May 1982; 1991; Bull 1994; Ewald 1994). In particular, the level of virulence depends largely on the probability and mechanism of transmission between hosts. If the rate of transmission is proportional to the reproductive rate of the pathogen inside a host then increased virulence can evolve (see Futuyma 1998: pp 549-551 for an introduction to the evolution of virulence). It would seem that as the number of virus particles in a host increases both the probability of transmission and virulence might increase as well. However, plant viruses have not been evaluated in this explicitly evolutionary context.

When exotic susceptible species, e.g. new crops, are introduced to that region, the virus is pathogenic because a state of stasis has not evolved in that virus-host combination. There are abundant examples of viruses being discovered because non-native crop plants are introduced into areas where they did not occur previously. Cacao and maize in Africa are two examples. In both instances viruses were discovered because they severely affected the new crop plants; however the viruses were not found to have any effects on their native host species (Bos 1981, Bos 1992, Bosque-Perez 2000, Storey and McClean 1930, Thresh 1958, Thresh 1980). Thus, to assume that viruses are pathogens of native species and infection therefore affects native species, is incorrect. Clearly, it is not appropriate to claim that all viruses reduce the fitness of their hosts. However, one panelist pointed out that it is important to remember that small reductions in host plant fitness (e.g. 10-15%) which would not be noticeable to the casual observer because plants do not die, or perhaps even show very severe symptoms, could still have large effects on the evolution and ecology of the hosts.

Appendix I of the Agency's background document nicely reviews studies examining the effects of virus infection on wild plant populations. For example, *Eupatorium makinoi* populations decreased in size (and were predicted to go extinct) following a virus epidemic (Funayama et al. 1997, 2001). Similarly, naturalized *Medicago* populations infected with alfalfa mosaic virus showed decreased competitive ability and decreased seed set relative to uninfected populations. As noted in Appendix I of the Agency's background document, these examples clearly indicate that viruses have the potential to limit wild (or naturalized) populations. This is relevant to the proposed exemption because these species are not currently weedy or invasive. However, as noted by the previous SAP, there are documented examples where virus infection confers positive characteristics on native species (Gibbs 1980).

Two recent studies have examined the fitness effect of PVCP-PIPs introgressed into wild genetic backgrounds. Fuchs et al. (2004) found that, in the presence of virus, virus-resistant transgenic squash had higher fitness than non-transgenic squash plants. Similarly, Sukopp et al. (2005) found that, in the presence of virus, wild beet hybrids with transgenic virus resistance outperformed their non-transgenic counterparts. This is in contrast with earlier work, by the same group using the same species, but different genetic material (Bartsch et al. 1996), cited by the previous Panel, which came to the opposite conclusion. This Panel suggested consideration of PVCP-PIPs should be on a case-by-case basis.

Another approach to evaluating the effect of viruses on plant population dynamics in natural ecosystems is to compare the weediness of species in their native range (where they co-occur with a suite of viral and other pathogens) and their introduced range (with fewer pathogens). Mitchell and Power (2003) showed that plants with proportionally fewer pathogens

in their introduced range were more likely to be invasive or noxious weeds. These results suggest that pathogens, including viruses, in some cases, may regulate their host plant populations.

Because there are currently few studies evaluating the effect of virus infection on plant population dynamics, it is useful to also consider the effects of fungal pathogens and insect herbivores on plant dynamics and plant community structure. Through the 1980's and early 1990's many ecologists doubted that insect herbivores had much effect on plant dynamics. This was largely due to the observation that most plants do not seem to experience much herbivory, together with the absence of studies specifically examining the effect of insects on plant dynamics (which is arguably exactly the situation for virus/plant interactions today). Studies suggesting that herbivory could have effects on plant population size (Louda and Potvin 1995; Guretzky and Louda 1997) and plant community composition (Carson and Root 1999, 2000) were initially a surprise to many, but it is now generally accepted that insect herbivores can have large modulating effects on plant population dynamics (Ricklefs 2002). Similarly, Burdon (1987), Alexander and Antonovics (1988), Fowler and Clay (1995), and Lively et al. (1995) all suggest that fungal pathogens can regulate plant population dynamics. By analogy, these studies suggest that plant populations could at least sometimes be regulated by virus infection. However, as noted by the previous SAP, positive effects due to virus infection have been noted (Gibbs 1980).

The current Panel's conclusions differed slightly from both the Agency and the October 13-15, 2004 FIFRA SAP reviewing PVCP-PIPs. Although both Appendix I of the current Agency background document and the previous Panel discussed literature suggesting that virus infection can lead to decreases in wild plant fitness and/or changes in population dynamics and community structure, both concluded that PVCP-PIPs will not have negative environmental effects. The Agency noted further that there is a scarcity of available data with which to address the potential impact of a PVCP-PIP on plant population dynamics, specifically, "The changes in plant population dynamics potentially introduced when a plant that is not weedy or invasive acquires virus resistance are likely to fall within the range of changes that happen naturally within plant communities without adverse effects." Some members of the current Panel argued that no empirical evidence is presented to support this opinion; in fact, much of the evidence that is presented is contrary to this opinion.

The majority of the October 2004 FIFRA SAP was of the opinion that "it would be unlikely that a plant population freed from viral pressure would give a plant species a competitive advantage." This is based on the accumulating understanding that viruses co-evolve with their hosts to reach an equilibrium in which neither is compromised (Lovisolo et al. 2003). The evidence presented in support of this view (Bartsch et al., 1996) has been refuted more recently by the same group using a more efficient PVCP-PIP construct (Sukopp et al., 2005). Some members of the current SAP agreed with the previous SAP. Other members of the current SAP believed, based on new information (Fuchs et al. 2004; Sukopp et al., 2005) not available to the the 2004 Panel, as well as EPA indicating a lack of data on this topic, that concluding that viruses typically have no effect on their wild plant hosts is not accurate. Because of the differing opinions among the current Panelists, and the general paucity of data, the Panel cautioned that further research is needed to provide stronger support to this particular issue.

One Panel member pointed out that conventionally-bred single-gene resistance has been used for decades with no reports of the resistance moving into wild populations and causing environmental harm. The Panel member further suggested that this observation indicated that PVCP-PIPs moving into wild populations would similarly cause no harm. Other Panel members countered that if such a resistance existed in the biodiversity of the species genome, it was probably already in the wild population and, thus, not likely to cause harm if it moved back into the wild population. The Panelist also pointed out that if the phenotype is resistant, it would not matter whether or not the gene movement was a transgene or plant-derived resistance gene, and therefore the previous sentence is not assessing effect, but type of gene. Other members countered that if such resistance existed in the biodiversity of the species genome, it was probably already in the wild population. Effect is what is important. The Panel could not identify any specific examples of introgression into wild populations of conventionally-bred resistance. These cases, if they exist, would likely be quite informative.

One Panel member suggested that exemptions should be given to crops that produce only very low fitness hybrids when they naturally cross with their wild relatives. However, it is known that favorable alleles (including, perhaps, a PVCP-PIP) can pass easily from one species to another through hybrid zones, even when the hybrids have very low fitness (Barton 1986). Genetic material can even flow between polyploid species that do not have homologous chromosomes but do have homoeologous chromosomes (chromosomes with partial homology) (Weissmann et al. 2005).

The Panel went on to discuss other possible ways in which the Agency could provide exemptions for crops with sexually compatible wild relatives. The Panel generally agreed that if highly effective biological containment and biological mitigation methods could be deployed concurrently with the PVCP-PIP, then it would be possible to exempt crops with sexually compatible wild relatives. This opinion is different from the opinion of the October 2004 FIFRA SAP. The current Panel concluded that this difference is probably due to advances in containment and mitigation strategies. For this reason, exemptions might be granted to any crop that hybridizes with a wild relative in the US, its possessions or territories, if the F_1 and BC (backcross) hybrids have very low fitness such that it is effectively lethal. Additionally, an exemption might be possible if specific genes for lowering fitness are in tandem constructs with the PVCP-PIP gene in such a way that they cannot readily segregate from each other. The Panel did not determine what level of effectiveness would be required but, it was agreed that stacked strategies would reduce the cumulative risk, and should be strongly considered.

In addition, the Panel encouraged the Agency to consider the geographic distribution of crops and their wild relatives when considering potential exemptions. For example, if a crop planted only in the continental US had a sexually compatible wild relative only in Guam, an exemption would be appropriate. Panel members disagreed about the degree of geographic separation necessary to grant exemptions. One member believed that separation within North America would be enough (especially if varieties bred for one geographic zone would not produce reproductive plants in the zone in which the compatible wild relative occurs); another believed that sexually compatible relatives anywhere within the continental US (for crops planted in this region) should preclude an exemption. No Panel member believed that if there

were a related species only in a distant US possession (e.g. Guam) that an exemption should be precluded, and the Panel suggested that the agency develop guidelines for petitioning to have geographic barriers taken into consideration for such crop species.

Question 2(c)

Please describe any additional factors beyond those listed above that the Agency could use to evaluate whether a PVCP-PIP meets (i) or (ii).

Panel Response

Since the Panel recommended that there be no general exemption for PVCP-PIP crops with sexually compatible wild relatives, the Panel did not discuss additional methods to determine the weedy, invasive, or threatened/endangered status of those relatives.

Question 3

Panel Summary - Viral Pathotypes, Novel Viral Interactions and Environmental Concerns

The Panel agreed that the likelihood for "novel interactions" is very low, and the environmental concerns that might result from potential novel viral interactions that could result from the use of PVCP-PIPs in the United States, its possessions or territories is lower than that which occurs naturally from mixed virus infections.

Please comment on the usefulness of the following criteria (i) and (ii) in correctly identifying PVCP-PIPs that present low risk with respect to environmental concerns associated with novel viral interactions. Please explain the basis for your answer, including whether the limitations imposed by the use of "viral pathotype," "naturally infect," "species," and "Unit in his 1/24/06 emailed States, its possessions, or territories" are necessary and/or sufficient. For example, could other parts of North America be included as part of criterion (i)?
(i)the viral pathotype used to create the PVCP-PIP has naturally infected plants in the United States, its possessions, or territories and
(ii) the viral pathotype used to create the PVCP-PIP naturally infects plants of the same species as that containing the PVCP-PIP.

Panel Response

The Panel used the definition given by the Agency: a "novel viral interaction" is an interaction (i.e., recombination, heterologous encapsidation, or synergy) between viral transgenes and an infecting virus involving viruses that would otherwise not be expected to interact in a mixed infection found in nature. The Panel agreed that the likelihood for "novel interactions" is very low, and the environmental concerns that might result from potential novel viral interactions that could result from using PVCP-PIPs in the United States, its possessions, or territories is lower than that which occurs naturally from mixed virus infections. The general consensus was that point (i) could be modified to state that if the sequence was engineered to express a protein,

the sequence should be from a virus originating in the United States, its possessions or territories. If the sequence was designed for PTGS, this restriction does not apply. Point (ii) is acceptable with the above modification regarding "sexually compatible" species.

The Panel discussed the various terms used in this question (i.e., "viral pathotype", "naturally infect", "species" and "United States") and concluded that "naturally infect" was acceptable as used by the Agency.

Regarding the use of "viral pathotype" there was not much discussion of this term. The Panel suggested that logic says that local or indigenous virus isolates, or those with significant sequence similarity, will be used to generate PVCP-PIPs. From what we know now, only those viruses with high sequence identity will be useful as sources of the PVCP-PIP transgene. A good example is the papaya ringspot virus (PRSV) transgenic resistance used in papayas in Hawaii. Only local virus isolate sequences are effective and therefore used in the transgenic papayas.

The Panel also commented that while the Agency addressed risks with the use of PVCP-PIPs, there was a lack of discussion on the benefits of using this technology. There was general discussion and agreement that while the risk with PVCP-PIPS is low, some mention of the benefits should be given. There was discussion that mixed virus infections are recognized as common, virus sequences (and proteins) are in higher concentration in virus-infected cells and that any potential for such interactions already occurs naturally in mixed infections. Virus resistance resulting from PVCP-PIPs would result in fewer virus infections and less risk.

Question 4 - Coat Proteins, Novel Viral Interactions and Environmental Concerns

Panel Summary

It is widely acknowledged that plant viruses have evolved and continue to evolve by a concept termed modular evolution coupled with the accumulation of point mutations. Modular evolution occurs by recombination and there is ample experimental and phylogenetic evidence that viruses naturally recombine. Given this, the Panel was charged with determining the risks associated with recombination between any form of a PVCP-PIP and an infecting virus and with creating criteria for exemptions. There are three biological entities in this interaction that affect recombination: the plant host, the virus from which the PVCP is derived, and the (super) infecting virus. It was concluded that any recombination events that can occur will be benign and no different than would or has occurred in nature.

The Panel was able to envision scenarios of potential (yet extremely low probability) recombinants in situations where a complete virus coat protein (CP) or any other virus protein is the PVCP. It is now known that many currently deployed PVCP-PIPs are likely virus resistant due to PTGS and not the result of CP expression. PTGS does not require CP or any other viral protein expression. Based on this scientific knowledge, the Panel created a decision flow-chart incorporating criteria for exemptions for consideration by the Agency. Briefly, any PIP that does not express a virus protein and is designed to trigger host PTGS, would be exempt. It was further recommended that all future virus control PIPS be generated to establish host PTGS

against the virus for which resistance is desired. For those cases in which the PIP is gene-expressing (protein producing), most can be exempted as well. There are a few scenarios, however, in which recombination may have an incrementally higher probability of creating a virus with new properties. The decision flow-chart identifies these potential situations and provides the Agency with the opportunity to review them. In conclusion, the Panel recommended the need for the Agency to have criteria to assess the level of risk relative to novel virus interactions.

Question 4 (a)

Please comment on the usefulness of the following criteria (i) and (ii) in allowing the Agency in its review of the product to identify correctly whether the PVCP-PIP presents low risk with respect to environmental concerns associated with novel viral interactions. Please explain the basis for your answer.
(i) the properties of the viral pathotype that are determined by the coat protein gene used to create the PVCP-PIP are substantially similar to the properties of a viral pathotype that naturally infects plants in the United States, its possessions, or territories, and the viral pathotype used to create the PVCP-PIP naturally infects plants of the same species as that containing the PVCP-PIP, or
(ii) viruses that naturally infect the plant containing the PVCP-PIP are unlikely to acquire the coat protein sequence through recombination and produce a viable virus with significantly different properties than either parent virus.

Panel Response

The Panel agreed that criteria 4 (a)(i) is unusable and cannot be re-written into a satisfactory form. The Panel understands the intent of the criteria; that is, the closer the PVCP-PIP is to other infecting viruses in terms of primary sequence, secondary and tertiary structure, amino acid sequence, phenotypes and virus regulatory activities conferred, the less likely that any recombination event would result in a new entity with unique properties and the more likely it should be exempt from further regulation. Specific issues included the need to define "properties". A partial but incomplete list of these properties is mentioned in the previous sentence. The Panel discussed the meaning of "substantially similar" as a criterion. The concept of a virus "quasispecies" was discussed relative to "substantially similar". The Panel could not satisfactorily define "substantially similar" to make it usable as a criterion. Given these issues, the Panel recommended that this criterion be deleted.

If the PIP did not express a CP, this criterion was not necessary. If the PIP does express a CP it should be sent through a draft decision flow chart that takes into account the relative likelihood and consequence of any recombination event between the PIP and a superinfecting virus.

The Panel had considerable discussion concerning the bifurcated nature of any PIP that has been and will likely be made. The four PVCP-PIPs already released were intentionally created to express CP based on the knowledge of the time. It is widely acknowledged that the underlying mechanism providing virus resistance in these cases is PTGS. PTGS does not require

CP expression; it is strictly triggered by a threshold amount of homologous RNA. The Panel was aware that the Agency's criteria under consideration addressed both the legacy PVCP-PIPs and any future PIPs. Thus, the remainder of the Panel's response addressed criteria based on whether the PIP expressed protein or not, as many of the regulatory issues are related to protein expression.

One Panel member noted that PTGS results in small RNA from the PIP and the infecting virus that could, in certain circumstances, be recombinatorial. The Panel recognized this possibility, but concluded that this minimal RNA would not confer a phenotype to the recombinant, would result in just a few nucleotide changes in a potential recombinant, and thus would be irrelevant. For this reason, the Panel recommended exempting any PIP that is not encoding a virus protein and is designed to induce PTGS.

There was some disagreement among the Panel whether a CP could confer a trait or phenotype independent of its other cognate viral proteins. Overall, the Panel agreed that some traits/phenotypes/functions are solely resident within the CP in the absence of interactions with other viral proteins and others not.

In terms of recombination, discussion centered on the likelihood, frequency, and consequences of virus recombination between the PIP and a virus infecting the PIP plant. While the discussion was wide ranging, it was repeatedly stated that the consequences of any recombination event are minimal. This conclusion was based on the fact that nearly every plant on the planet is harboring multiple virus infections with both closely related and taxonomically distinct viruses, with essentially no new viruses emerging with substantially different properties and causing wide pandemics or undesirable environmental effects. Since we have had the ability to identify viruses, essentially all new viruses that have emerged as major pathogens pre-existed and have emerged due to altered host genotypes, cultural practices, or change in climate. This conclusion is also strongly supported by the results of 10 years or more of field experience with transgenic crops. If the PIP did not encode a protein, the likelihood of any recombinant occurring with new pathological properties was further diminished to the point that if the PIP did not generate a CP, this criterion is not necessary. Finally, one Panel member stated that the frequency of recombination was somewhat irrelevant; it is the consequence of the recombination that was much more important than frequency.

In terms of any relationship that might exist between the length of the PIP sequence and the frequency of recombination with a second virus, it was concluded that the shorter the PIP sequence, the less likely recombination is to occur. However, there are so many other factors involved in recombination like AU/AT content, hairpin structures, promoters and others, that the complexity precludes development of an effective criterion regarding the length of the PIP sequence.

Question 4(b)

Please comment on the usefulness of the analyses described above for evaluating whether a PVCP-PIP meets (i) or (ii). Please describe any additional factors that the Agency could use

in this evaluation (e.g., consideration of whether the plant virus species has an inherently low natural recombination frequency with respect to the coat protein gene).

Panel Response

There are some recent reports of examples of recombination occurring in the absence of replication (Gallei et al. 2004; Gmyl and Agol 2005). In the absence of data relating to plant viruses, no consideration of this issue was made in the recommendation as presented in Figure 1. The figure presents a flowchart for creating decision criteria. The first point to consider is whether the PIP expresses protein or not. If the PIP does not express protein, regardless of its phylogenetic relationship or degree of homology with the superinfecting virus, it should be exempt. If the PIP does express a complete viral protein, then it would go through the flow chart to assess the likelihood of recombination and consequences of a recombination event.

Three "organisms" are considered in Figure 1: the host plant which contains the PVCP-PIP, the PVCP-PIP sequence which is the donor to any recombinational event and the superinfecting viral sequence which is a virus that can infect a plant containing a PVCP-PIP; this would be the recipient of any recombinational event. The initial question is "Does the PVCP-PIP produce a protein?" The answer "No" assumes that the protection mechanism is via PTGS and consideration should address the points raised in response to Question 6. The answer "Yes" leads to the question "Is the protein complete?" If it is not complete the question arises as to whether recombination of the sequence could lead to a significant change in the properties of the recombinant over the original properties of the superinfecting virus. Significant changes include increase in pathogenicity, increase of host range or change of vector. Moreover, because most viral CPs are multifunctional (Callaway et al. 2001), the majority of envisionable recombination events that could occur would be expected to yield a chimeric CP. A chimeric CP would almost always result in a lethal mutant and thus a virus that would be unable to survive under wild type selection. Further, as the CP is required for invertebrate transmission of the majority of plant viruses, vector specificity and competency would be abolished by minor or major replacements of amino acids that alter capsid structure and/or key amino acids of the CP that are specifically involved in vector-mediated transmission.

The presence of a complete protein or of an incomplete protein that contains significant amino acid sequences leads to an analysis of whether the PCVP-PIP sequence contains features significant in RNA or DNA recombination. For RNA viruses, these include the presence of AU-rich regions, and for both types, the presence of hairpin sequences and the presence of nucleic acid sequences homologous to analogous regions of the superinfecting virus genome sequence. However, the Panel was uncertain as to whether these sequences have to be present on the donor and/or on the recipient. This needs to be determined by literature search before the flow chart is finalized.

The answers to the questions in Figure 1 lead to either a recommendation for exemption or the need for further consideration. The latter leads to Figure 2, which takes account of the type of RNA-dependent RNA polymerase (RdRps) encoded by the superinfecting virus and the compartmentalization of its site of replication.

As noted in response to Question 4, the RdRps of plant viruses differ in the propensity for initiating recombination, whereas small plant DNA viruses employ host DNA polymerases, which have built-in repair mechanisms resulting in high fidelity copying of the template. The great majority of recombinational events involve replication of RNA and DNA, and the donor and recipient should be in close association. Thus, there is a question as to the possible association of the donor and recipient during RNA replication.

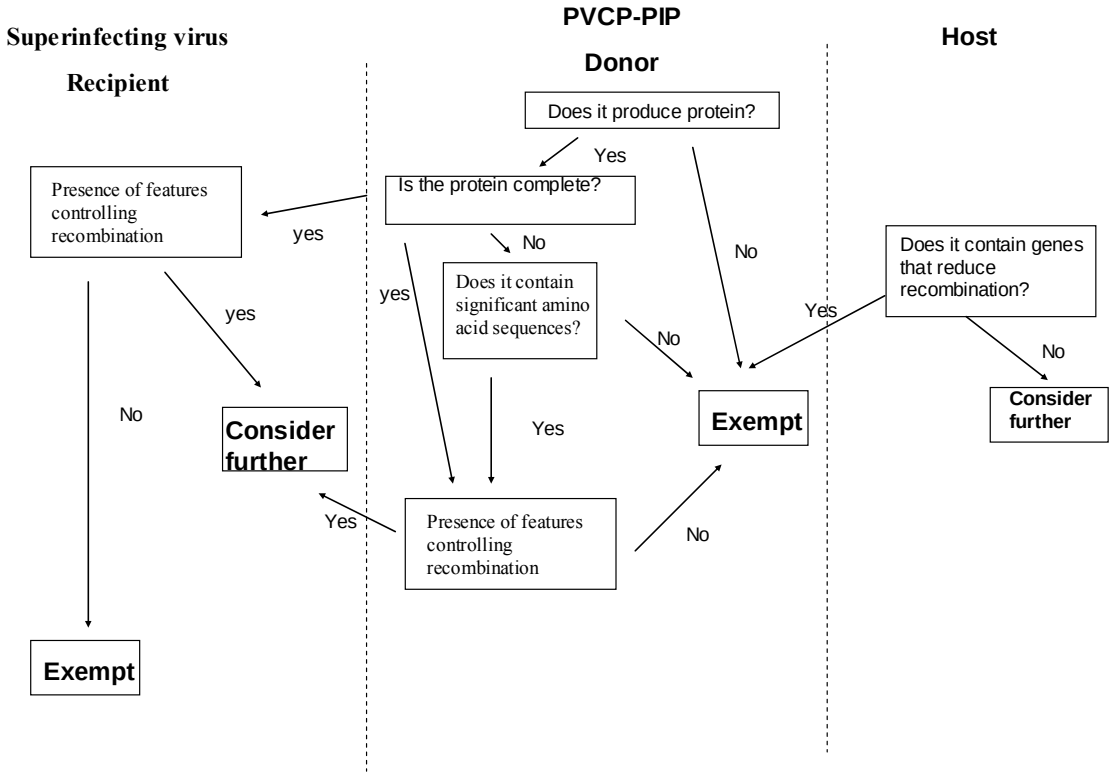


Figure 1. Criteria For Exemption

The Panel discussed whether the Agency should encourage all future PIPs to be based on PTGS. This prompted the discussion of whether PTGS increased or decreased the likelihood of recombination as noted in response to Question 4a above. It was decided that this question might be premature given the lack of a complete understanding of PTGS, but at this time, based on the idea of reduced levels and size of viral RNA or DNA (particularly when the replication initiator protein (Rep) is the target of PTGS), recombination should be greatly repressed in PTGS plants.

One Panel member noted that all RNA viruses have one of three replicase modules, each with a different inherent capacity to recombine. Another member questioned the idea of using this as a criterion as it is difficult to predict which second virus may infect the PIP plant. This member also questioned the inclusion of this knowledge as part of the flow chart. The member who brought up the information on the three types of viral replicase also noted that one could infer the likelihood of two viruses recombining based on what intracellular site of replication is used. It was decided that at this time, a potential criteria based on this property would be too vague.

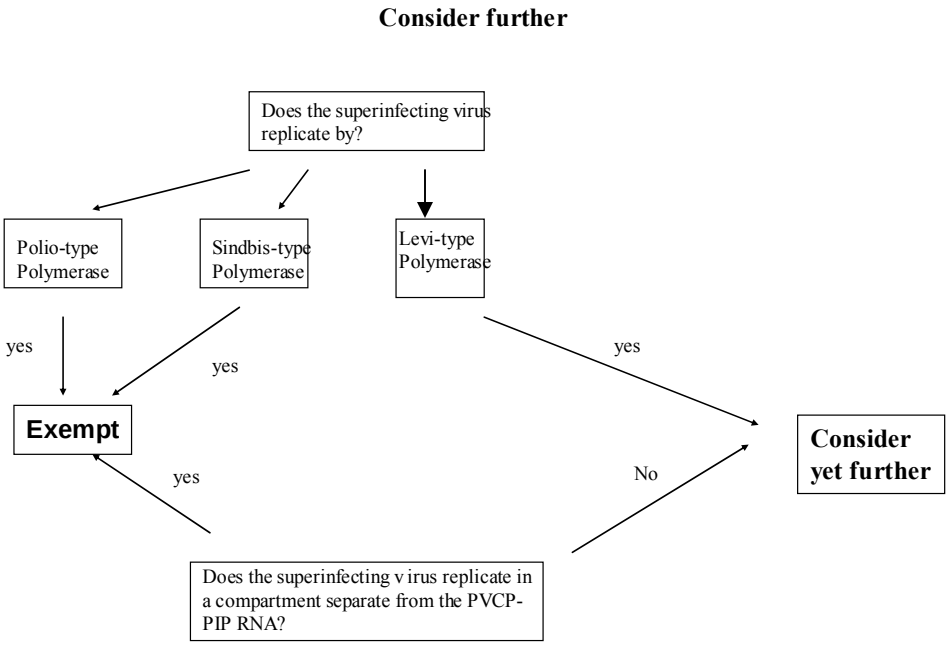


Figure 2. Flow Chart For Recombination of PVCP-PIPs

Question 5 - Nontarget and Human Non-dietary Exposure to PVC-proteins

Panel Summary

The Agency is currently considering whether PVCP-PIPs present low risk with respect to nontarget and human non-dietary exposures to PVCP-proteins if the genetic material (i) encodes a protein that is minimally modified from a coat protein from a virus that naturally infects plants or (ii) produces no protein.

The Agency has presented their process nicely and has done commendable work to determine appropriate factors to identify risk associated with PVCP-PIPs. VCPs are naturally present in the environment and no adverse effects to humans or non-targets have been reported. Initially, this leads to the thought that risks will be minimal. Even so, the Agency is correct to proceed with care since, unlike chemical pest control strategies whereby active chemicals are applied and dissipate with a known (usually short) half-life, genes transfected into plants have the potential to persist in the environment through continued production of the pesticidal material. The Panel agreed that dermal exposure to PVCP-PIPs poses far lower potential risk than does inhalation exposure. Additionally, there may also be indirect ecological effects (such as altered food sources, vegetative cover, or microbial communities) that would warrant evaluation.

Please comment on the usefulness of the above factors in allowing the Agency to identify correctly those PVCP-PIPs that present low risk with respect to nontarget and human non-dietary exposure to PVC-proteins.

Allergies: Some Panel members were concerned with the Agency's position that no plant virus coat proteins have been identified as allergens. Even if PVCP-PIPs are of natural sequence and structure, the probability of expression in all plant parts, *specifically pollens*, may be problematic in risk assessments. This potential risk is due to the fact that pollens are a major source of inhalant allergens, so plants with pollen that is not inhaled should pose less concern. Moreover, the entire population in a certain climatic region is exposed to pollen via the airways and other mucous membranes such as the conjunctiva. Plant-derived particles are known to cause allergies in areas of intense mechanical harvesting (e.g. cotton production in West TX or CA). Introduction of new proteins to pollens and other plant materials may have the potential to cause problems, and consideration by the Agency is warranted. As a guide, the Agency should consider that there are three major ways that plant proteins can induce and elicit IgE-mediated allergies: pollen allergies, allergies to proteins in small airborne particles, and food allergies (Table 1). The latter does not address the question posed, but in order to understand the implications of PVCP inhalation, factors controlling the onset of airborne allergies can be described in the following way:

> **1) Allergies to Pollen:** Pollen grains should not exceed a certain aerodynamic size in order to be a major source of allergies. In other words, if they become too large (such as pine pollen), they are no longer airborne and cannot be inhaled (Table 1). Even though the most important allergens are characterized and available in recombinant forms, it is unknown whether the allergenic proteins always constitute a large part of the protein fraction in a pollen grain. These allergies may affect 10-15% of the population.
>
> **2) Allergies to Proteins in Small Airborne Particles:** Such particles are generated in industrial processing plants and are not limited to food industries. The creation of small particles and maintenance of a high exposure often require large volumes and strong mechanical forces. Thus, most of these allergies are occupational, such as allergies to components in flour (bakers' asthma), latex gloves, soy bean dust, etc. Most described individual allergenic proteins seem to be abundant proteins of the materials. While the incidence of specific allergies may be considerable in a few settings

Table 1. What factors may lead to (or modulate) an IgE-mediated immune response?

	Pollen Allergies	Occupational Allergies	Food Allergies
Protein Structure	Suggestions that allergenic proteins fall in few, out of a large number, of structural families, but no clear evidence that this is directly related to allergenicity.		
Dose	Ragweed pollen allergen 60 ng pr. season	Clearly higher concentrations. Optimal single exposure 10 μgram in one dose with optimal adjuvant (Riedl-2005, human data)	Not known. Probably mg to gram dosages.
Abundance Of Protein In Allergenic Source	Not known	Likely high	Likely high
Administration Form (To Reach The Mucous Membranes)	>50 micrometer for rhinitis less for asthma	>50 micrometer for rhinitis: less for asthma	Digestibility a factor, however, food allergens may be absorbed via the oral cavity (Dirks-2005). Many food allergens cross-react with inhalant allergens as primary sensitizers.
Adjuvants: Alum (Mice); Parasitic Extracts; Diesel Exhaust Particles; Lipid Fractions (PGD2-Like: Phyto-Prostans) Of Pollen	Adjuvant effects of diesel exhaust particles have been demonstrated in man.	Adjuvant effects of diesel exhaust particles have been demonstrated in man.	Adjuvant effects (of cholera toxin) leading to food allergies have been demonstrated in rodents.
Atopic Status, Genetic Susceptibility	Yes	Yes	Yes
Previous Allergies	Yes	Not known	Not known
Other Diseases	Asthma	Asthma	Not known

and industries, the overall prevalence of these allergies in the general population is low, probably well below 1%.

Other Panel members felt that, unless there is evidence that PCVP-PIPs are expressed on the surface of pollen grains in a manner different from expression in wild-type plants, the risk of increased allergy from exposure to pollen is non-existent. Further, these Panel members felt that the risk from other particulate matter is no different for PCVP-containing plants than from plants with virus infections.

Overall Protein Expression: The Panel expressed concern that besides possible quantitative or qualitative changes in the expression of the PVCP-PIP proteins, the changed infectivity status of the plant may also induce changes in the overall protein expression pattern of the plant. Thus, in various tissues of the plant, plant proteins that have been identified as allergens may be expressed to a different, and in some cases, higher extent compared to a non-infected or a virus-infected plant without PVCP-PIP. In particular, pathogenesis-related (PR) proteins are known to be very inducible, and their expression levels may vary many-fold. Several pathogenesis-related proteins have been described as allergens (Breiteneder et al. 2000 and 2004), most notably the major birch pollen protein Bet v1 [Breiteneder et al. 1989). An increased expression of PR-proteins in pollen could increase both the risk of sensitization and the risk of elicitation of allergic reactions. For this reason the question of changes in expression of known plant allergens has been put into a decision tree for evaluating PVCP-PIPs risks.

Possible Safety of VCP Containing Pollens: The Panel believed that the potential for inhalation exposures to viruses in pollens is adequately addressed and of low risk based on knowledge that a number of plant viruses (e.g., at least four different virus families) are naturally spread in and on pollen (Table 2). This route of virus transmission is the exception, not the rule. Even so, limited virus presence in plant pollens does not preclude the concern for allergies associated with a broad spectrum of PVCP-PIPs that could be newly added to pollens.

Table 2. Pollen Transmitted Viruses (Hull 2001)

Virus Genus	**Virus**	**Plant Host**
Ilarvirus	*Blueberry shock virus*	Blueberry Fruit
	Prune dwarf virus	Stone Fruit
	Prunus necrotic ringspot virus	Stone Fruit
	Tobacco ringspot virus	Various
Nepovirus	*Artichoke yellow ringspot virus*	Artichoke
	Blueberry mottle virus	Blueberry
	Cherry leafroll virus	Walnut, *Betula (birch)*
Sobemovirus	*Sowbane mosaic virus*	*Chenopodium*
Ideavirus	*Raspberry bushy dwarf virus*	Raspberry

The Panel also believed that naturally occurring VCP would be produced in much larger quantities through natural infestations than in plants containing VCP genetic constructs. For

example, tobacco mosaic virus produces gram quantities of VCP in seriously infected plants. Quantitative VCP data from representative transgenic plants is necessary to verify any comparison of relative protein production.

Concern for Multiple Constructs

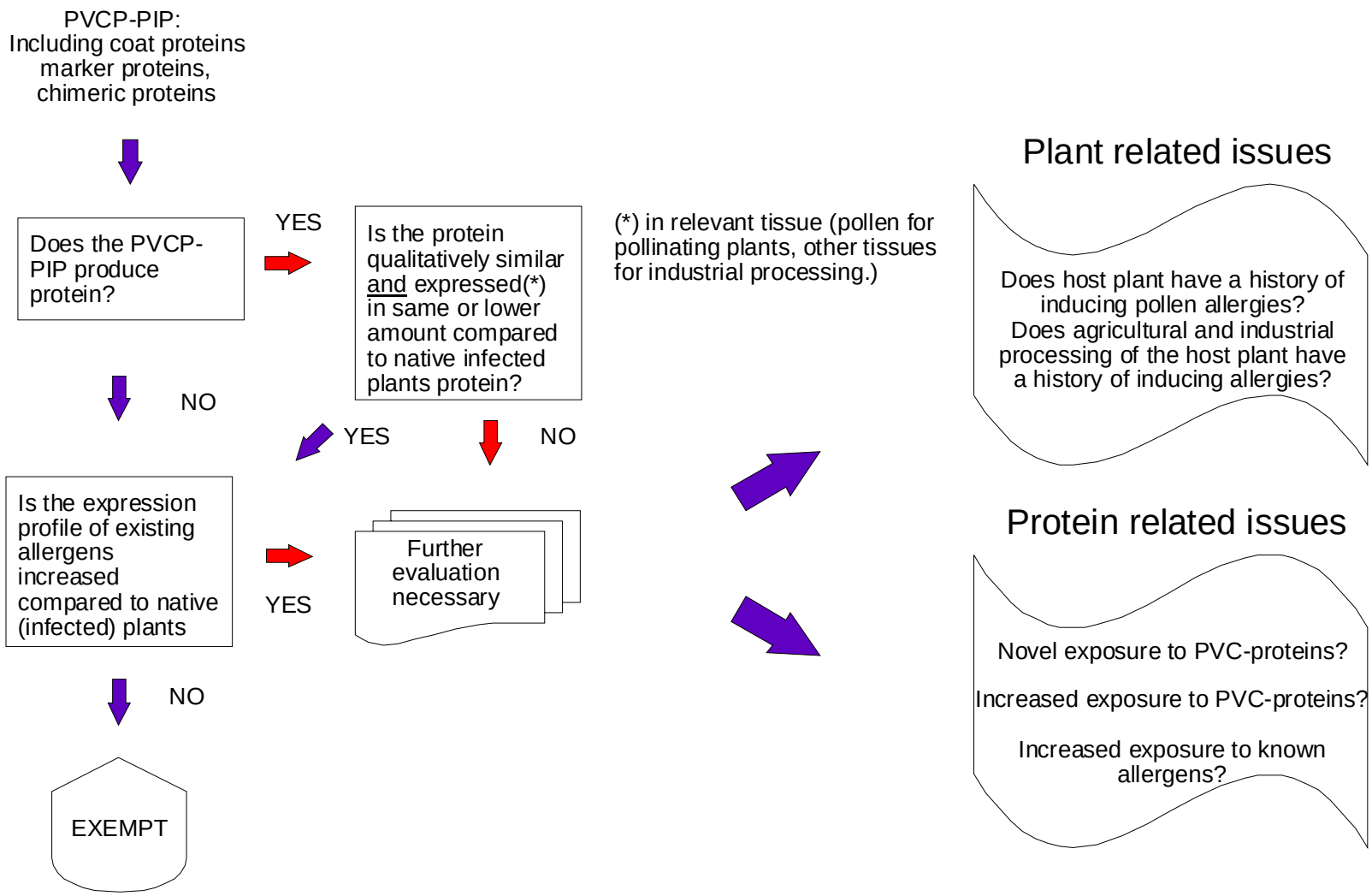
Some members of the Panel were concerned that multiple constructs could be exempted from review by the Agency. While it will not always be essential for review, the potential to review such multiple constructs should be considered. For example, *Bacillus thuringensis I* (BTI) toxicity in combination with certain natural substrates (e.g., kudzu/tulapia combinations) alter the BTI bacteria by turning on genes to make them more toxic (Reeves 1985) . This example and other situations demonstrate the need to evaluate chemical mixtures or mixtures of pesticidal proteins. Inclusion of several gene constructs may produce multiple proteins in foods or alter "natural" protein production in plants. These situations may exacerbate any allergic responses by consumers or alter the nutritional value of food crops. EPA evaluations should consider effects of multiple constructs of PVCP-PIPs introduced in transgenic plants. Other Panel members believed that this situation was no different than is likely to occur in nature, where a plant might be infected by multiple unrelated viruses.

Appropriateness of Factors for Assessing Low Risk

Based on the allergenic potential of PVCP-PIPS, the Panel concluded that the factors presented by the Agency are useful, yet incomplete to allow regulatory exemption of these PVCP-PIPs**.** Thus, the Panel proposed a decision tree (Figure 3) that adds to the Agency's proposed factors. The Panel believed that this decision tree can be merged with Figure 2 for gene flow/recombination that was presented in Question 4. The text below expands on the decision tree and provides guidance for assessment techniques that can be incorporated in the event that an exemption is not warranted. The Panel also believed that additions are needed to the Agency's proposed factors involving allergenic risks to humans and determining effects on important, unevaluated non-target species.

It is envisaged that under certain circumstances (i.e., PVCP-PIP containing non-coding regions) review of the PVCP-PIP's genetic material is appropriate for assessment of protein production. However, there are other instances where protein expression cannot be fully assessed by review of genetic material. A full assessment of protein production in the plant would need to be assessed under the conditions listed below. Assessment of protein production would require the development and usage of standardized methodologies for protein analysis (e.g., ELISA). It should be noted that standardization of protein assessments needs to be sufficiently adaptable to allow emerging highly-sensitive protein identification methods to be used by registrants.

Figure 3. Decision tree for evaluating VCP-PIPs risks as related to Questions 5 and 9.



Natural Sequences: Correct identification of low risk PIPs should include existing protein structures from viruses that exist in plant species that occur in the US or that are derived from natural viruses considered to pose significant risk to US agricultural production. The Agency has focused on amino acid sequence and molecular weight as criteria. These are important criteria and should be considered, but when evaluating structures that fall within the "natural variation" of plant viruses, acceptable proteins should contain naturally occurring VCP amino acid (AA) sequences. This requirement should not contradict gene alterations proposed in Panel responses to earlier questions, as their proposed modifications address gene regions that do not actually code for protein.

Minimal Modification of Naturally Occurring Proteins: The concerns of protein incorporation into pollens is also a risk to be evaluated for VCP-proteins that fall into this classification. When determining which minimally altered protein structures are acceptable, it is important to note, as was discussed in response to Question 4, that protein structure is important, and that a small number of amino acid differences in critical regions can greatly alter three dimensional protein structure, thereby altering protein function. For this reason, the Panel recommended evaluation of three dimensional structure (such as the occurrence of regions of alpha helix or beta sheets) for any protein that is not a natural protein or fragment of a natural protein. If VCP structures are not reasonably well defined or cannot be modeled with commercially available molecular modeling software (such as Jaguar: Schrodinger, Inc.), the Agency may need data from a variety of standard analytical natural product chemistry techniques to define structure (HPLC MS/MS and NMR) in combination with sequence data.

Some Panel members felt that the importance of structure in protein function/effect can be seen in the chemistry of natural toxins. The properties of these natural toxins emphasize the importance of structure-activity relationships in evaluating protein effects. Specifically, changes in the planar nature and charge are of great importance in terms of the ability of a gene to express its function and potential environmental effects of any protein it expresses. Such examples include Blue Box Jelly Fish Toxin and Metallothioneins. Thus, protein chemistry, albeit amino acid chemistry in terms of toxicology, can be complex. Other Panel members felt that protein toxins are not an appropriate model for assessing the effect of minor amino acid changes on potential allergenicity.

Cases of No Protein Production: The verification of no protein production will require rigorous evaluations of protein content in plant materials. Some Panel members expressed concern that changes in native protein expression in plants or in environmental conditions would initiate PIP protein production.

The Panel believed that documenting "no protein expression" presents technical difficulties. To demonstrate that no protein is produced, rigorous methodological standardization of quality control may be necessary for acceptable assay systems. This requirement would present issues of sensitivity and accuracy. Some Panel members felt that the degree of concern would depend on the nature of the genetic construct involved.

The notion of no protein production is a serious issue, because the concentration of protein exposure required to induce allergic reactions is unknown. Furthermore, the concentration appears to be somewhat allergen-protein specific. Thus, developing a protein concentration threshold would also not be sufficient.

For PVCP-PIPs containing genetic material that includes coding sequence or are designed for protein/peptide-induced gene silencing, some Panel members suggested that it should be assumed that proteins or small poly-peptides will be produced and consider criteria to assess exposure consequence of that protein. Other Panel members felt that the absence of promoters and ribosome binding sites should be taken into consideration as factors ameliorating concern.

Case of VCP Range of Natural Variation: The Agency is considering a criterion that describes PVC-proteins that are within the range of natural variation of the virus and therefore have a long history of safe non-target and human exposure. Historically, virus-infected plants have been a part of the human and domestic animal food supply without adverse human or animal health effects. The Panel agreed that exposure of PVC proteins generated in PVCP-PIPs that are identical with or within the range of natural variation of plant virus coat proteins would not alter the risk of toxicity and allergenicity. To assess whether the PVCP-PIPs are within the range of natural variation of plant virus coat proteins, prior knowledge of the natural variation limits of the individual PVC proteins is required.

Case of Long History of Safe Exposure: The Panel agreed that increased frequency of atopy in humans does raise concerns regarding the Agency's conclusion of low risk based upon their position that there is a long history of safe non-target and human exposure to PVCP-PIP proteins that are within the range of natural variation of the virus. In industrialized countries, up to 40% of the population are sensitized, i.e., have developed an IgE antibody response to allergens (Holt et al. 2005). It is currently not known whether natural variations of VCP-proteins have a long history of safety in an atopic population.

Case of PVCP-PIP Tissue Expression: The Agency could consider other criteria to be included for assessment exemption such as tissue expression. While plant viruses systemically infect plant tissues, there is tissue specific regionalization of viruses. Therefore PVCs would be restricted within certain compartments. Transgenic expression of some PVCP-PIPs would promote PVC expression in different plant tissues relative to what would naturally occur (i.e., all cells). This could lead to heightened levels of PVCs in certain tissues (i.e., pollen grains) and the effects (specifically to allergenicity) are not yet known. This has implications for non-dietary exposure of plant proteins. In some instances, PVCPs' natural exposure route may be via oral ingestion. However, genetically modified expression of PVCP-PIPs would lead to the presence of PVCs in other plant compartments such as pollen grains which lead to other sites of exposure including respiratory and cutaneous surfaces.

In Cases of Protein Production: Some Panel members expressed concern over potential effects on pollinators. This possibility has not been completely evaluated. Other Panel members felt

that a history of exposure by pollinators to naturally infected plants can be taken as indicating that there are no novel risks.

When considering risks of toxins or toxicants to humans or non-targets, exposure intensity, frequency, and duration across landscapes are important considerations. Indirect effects on non-target species should be considered just as in risk assessments for chemical insecticides. Lethality is unlikely to be a measurable endpoint from direct toxicity. Specifically, the current risk assessment has used a process that has assessed risk using mouse, fish and other appropriate models and has generally reached the conclusion that PVCP-PIPS are not toxic and do not pose a major risk to the environment. There are, however, possible concerns with non-traditional non-target organisms. If protein sequences or structures are determined to be altered significantly from natural proteins, the Agency should consider toxicity screens to determine adverse effects (e.g., mammals, fish, invertebrates) as well as cytotoxicity screens. As a first line of evaluation, the Panel suggested the mouse bioassay approach presented by EPA and a possible link with other routine acute screening bioassays.

Other Factors. In the case where protein alterations are considered to have occurred, several topics may need clarification or consideration.

- There is a reasonable possibility for detrimental "real world" interactions of the protein with other chemicals in the environment. For example BTI and carbamates were more than additively toxic to non-targets and insects. This may be important in terms of non-target risks or allergenic potential.

- Only freshwater organisms were considered, not estuarine or marine organisms. Many of these proposed crops will be grown in the coastal zone where the majority of these populations live. The Agency also needs to include bacteria and phytoplankton interactions (e.g., microbial loop community) as part of the risk assessment process.

- Sublethal effects were not observed in the risk assessment. However, sublethal effects should be considered such as indirect effect of proteins/amino acids in terms of food quality issues. It should be noted that some sublethal effects may be beneficial. Both *BTI*, *Bacillus sphaericus* and *Bacillus kurstaki* increased egg production in copepods because the invertebrates were able to use the protein/lipid as a food source to increase reproductive output (Scott and Williams 1986; Scott et al. 1987; Banks 1988; Dee 1988; Chandler 1989).

- PVCP-PIPs may not be directly toxic, but their effect on the quality of the food source into which they were inserted may be important. For example, indirect effects of chronic atrazine exposure resulted in less nutritious algae which affected bivalves, resulting in reproductive failure (ecological death) (DeLorenzo et al. 2001; Downing et al. 2004; Pennington et al. 2001; Pennington and Scott 2001, Lawton 2001; Pennington 2002). Effects on protein/amino acid structure could be an important consideration in chronic exposure.

- Retention ponds for agricultural runoff may be of concern if plants or plant parts incorporating PVCP-PIPs are used as food. Protein sorption to abiotic materials that are subsequently aerosolized should be considered. Effects in plants that have a shorter half-life may be of concern. For example, bacteria and aquatic plants like Harmful Algal Bloom organisms may be affected as plant species. The Agency needs to determine if there is a greater potential for protein expression to be observed in these species.

Question 6a

Please identify any characteristics of a PVCP-PIP construct that would indicate it is unlikely to produce PVC-protein. Please discuss the likelihood that protein production could nevertheless occur from constructs with these characteristics (i) in some tissues, (ii) at some life stages, (iii) under some environmental conditions, (iv) in the case of suppression of gene silencing, or (v) under any other circumstances. For example, how likely is PVC-protein production from a construct containing an inverted repeat of the coat protein gene (e.g., Mitter et al. 2003) or from a construct lacking a start codon (AUG sequence) and/or a ribosome binding site on the expressed RNA?

Panel Response

PTGS is a highly desirable strategy for generating virus resistant plants for several reasons. First, this strategy is based on a natural plant defense mechanism against viruses. Second, transgenes can easily be designed to produce only RNA, not protein. Third, PTGS gives stronger resistance than protein-based methods. Because of all these advantages, this is the premier virus resistance strategy for use today and into the future.

The Panel agreed that there were two types of PTGS-inducing plant transgene insertions that could be safely determined to have no protein expression regardless of plant tissue, developmental stage, environmental conditions, or exposure to virally-encoded suppressors of PTGS: 1) transgene insertions where the transcribed segment lacks an initiator methionine codon (AUG), for example through a base substitution mutation or through a 5' truncation of the protein-coding sequence, and 2) insertions of transcribed inverted repeat (IR) constructs that constitutively produce transcripts that are folded into double-stranded RNA (dsRNA)-like "hairpin" RNAs (hpRNA) as the immediate product of transgene transcription.

In the case of the IR type of transgene, even if the IR segment retained an initiation codon it would still be unable to produce protein because; 1) there is efficient cleavage of the dsRNA transcript by dicer ribonucleases, and 2) the initiation codon and its ribosome binding context are sequestered from the protein synthesis machinery by the base-paired secondary structure of the dsRNA. Thus, even if an IR transgene-containing plant was exposed to suppressors of PTGS that block early stages of PTGS (Qu and Morris, 2005), the plant would still be unable to produce protein from the transgene IR segment due to the secondary structure of the dsRNA.

In some cases a transgene construct originally designed as a transcribed sense segment or a transcribed antisense segment becomes integrated in the plant genome as an inverted repeat

array that constitutively produces dsRNA. Conversely, in some cases a transgene construct originally designed as a transcribed inverted repeat becomes integrated in the plant genome in a rearranged form or in a multi-copy array where dsRNA is produced indirectly (as noted in response Question 6B), rather than constitutively. Therefore, whether a transgenic plant falls into the IR transgene category must be determined by the nature of the transgene insertion structure in the plant genome rather than by the nature of the original transgene design. The structure of the transgene insertion in the plant genome can be determined by Southern blot assays.

One Panel member noted that, in some rare cases, naturally occurring protein coding regions initiate with AUU rather than AUG, so if the transgene segment was derived from such a non-canonical protein-coding region, the alternative initiation codon would need to be mutated to abrogate protein expression.

Question 6b

Assuming a PVCP-PIP construct does not possess any characteristics that would indicate a low likelihood of protein expression but PVC-protein is not detected in plants containing the PVCP-PIP, presumably because virus resistance is conferred through RNA, please comment on the likelihood and expected quantity of both RNA and protein that would be present (i) only transiently, (ii) only in certain tissues, (iii) only at certain life stages, (iv) only under certain environmental conditions, or (v) in the case of suppression of gene silencing? How likely is suppression of gene silencing to occur in the environment over time?

Panel Response

These types of PVCP-PIP plants carry sense or antisense transgenes that encode an open reading frame for either complete protein or fragments of viral coat proteins. These transgenes are constitutively silenced possibly because they form a complex locus of direct or inverted repeat arrays, or they trigger the action of the plant RNA-dependent RNA polymerase enzymes, leading to dsRNA production (Vaistij et al, 2002). However, PTGS of these transgenes might be less effective at the young seedling stages (Mallory et al., 2001), in the meristematic (Voinnet et al., 1998) and root tissues (Andika et al., 2005), at low temperatures (Szittya et al. 2003), or after infection by heterologous viruses that encode silencing suppressors (Brigneti et al., 1998). It should be pointed out that PTGS does work in roots, as has been shown in lupins (Uhde-Stone et al., 2005) and *Medicago truncatula* (Ivashuta et al., 2005), and at low temperatures (Sos-Hegedus et al., 2005). In the case of a transcribed antisense transgene insertion that retained an initiation codon for protein synthesis on the "upside-down" strand, there could still be sense-strand RNA transcription from a nearby endogenous promoter. Therefore an antisense construct cannot be excluded from the potential to express protein under conditions that suppress PTGS. Given the wide variety of conditions that can modulate the transition from PTGS to no PTGS for non-IR transgenes, for example the unpredictable nature of progressive promoter cytosine methylation that reduces transcription below the level needed for PTGS induction, it is likely that a non-IR transgene insertion that retains an initiation codon for protein synthesis will make at

least a low level of protein in at least some plant tissues over the course of its development, especially in the field where there is exposure to environmental extremes and virus infections. Thus, these PVCP-PIP plants may accumulate virus-derived mRNA and proteins in these situations. However, the level of the viral coat protein and mRNA will be many-fold lower than that in non-transgenic plants infected with the virus. In addition, PTGS-based virus resistance requires greater than 90% RNA sequence homology between the PVCP-PIP transgene and the target virus, indicating that the viral mRNA and protein produced in PVCP-PIP plants will be nearly identical to the viral pathotype that occurs in the United States. Because of low levels of accumulation and sequence identity to the natural viral pathotypes, the Panel concluded that these PVCP-PIPs pose similarly low risks as the hpRNA-based PVCP-PIP plants.

Question 6c

Please identify conditions under which protein detection methods should be conducted to determine whether PVC-protein is produced from the PVCP-PIP. For example, how many replicates and what particular tissues, life stages, and/or environmental conditions should be tested?

Panel Response

To determine if PTGS-based PVCP-PIP plants have the potential to produce proteins, the most effective test is to use viral suppression of PTGS. In this type of assay, the PVCP-PIP plants are infected with viruses from the potyvirus, cucumovirus, and tombusvirus genera. These viruses encode different classes of PTGS suppressor proteins that target different points in the pathway: potyvirus HC-Pro, cucumovirus 2b, and tombusvirus P19. Protein and RNA are then extracted from the infected plant tissue and assayed for the presence of the PVCP-PIP accumulated full-length RNA and protein. Standard tests for protein detection are ELISA and immunoblot ("Western" blot) analyses with specific antibodies. Triplicate experiments should be sufficient to determine that the results of these tests are reproducible. However, very low levels of protein expression would escape detection by these methods. Another potentially more sensitive assay for loss of PTGS is detection of accumulated full-length RNA by RNA gel blot or reverse-transcriptase PCR.

Question 6d

Compared with protein-mediated virus resistance, how does RNA-mediated virus resistance (e.g., during PTGS) affect the likelihood and possible environmental impact of (i) gene flow of a PVCP-PIP transgene and (ii) recombination of an infecting virus with a PVCP-PIP transgene or RNA transcript.

Panel Response

Based on our current scientific information, a PTGS transgene should have the same likelihood of gene flow into new plant species as a protein-mediated virus resistance transgene. Also, in terms of conferring a potentially advantageous virus resistance trait on a wild or weedy

relative of the original transgenic species, PTGS and protein-mediated virus resistance transgenes should have the same impact unless one type of transgene confers a much stronger degree of resistance than the other.

It is currently not known whether cleaved RNAs resulting from PTGS inhibit or stimulate recombination with infecting viral RNA. However, recombination between a full-length viral RNA and a cleaved small RNA resulting from PTGS would yield a truncated non-functional RNA. Therefore, a PTGS transgene poses negligible potential to yield novel recombinant viruses.

Question 7

7(a). What is the potential for novel human exposure to a PVC-protein when it is expressed in food from a plant species that the virus used to create the PVCP-PIP does not naturally infect (assuming that the virus naturally infects another *food* plant species)? What is the potential for allergenicity to be associated with such PVC-proteins? How would use of a small segment of such a protein (e.g., to achieve gene expression) affect relative concern for allergenicity?

7(b) Please comment on the likelihood that PVC-proteins containing terminal deletions are within the range of natural variation of plant virus coat proteins. What is the likelihood such truncated proteins would have increased toxicity or allergenicity relative to the corresponding full-length plant virus coat protein? What relevance does the size of the deletion have to this issue? What relevance does deletion at the C-terminus versus N-terminus have to this issue?

7(c) Please comment on the likelihood that a PVC-protein modified by an additional methionine at the N- or C-terminus would have increased toxicity or allergenicity relative to the corresponding unmodified plant virus coat protein. What relevance does the terminus at which the amino acid is added have to this issue? Of what relevance is the particular amino acid added? Of what relevance is the number of additional amino acids?

7(d) Please identify type(s) of protein modification(s) (e.g., internal deletions, amino acid substitutions, addition of certain amino acid residues) that could be introduced without resulting in a PVC-protein that would have increased toxicity or allergenicity relative to the corresponding unmodified plant virus coat protein, e.g., because the changes are expected to be within the range of natural variation for all virus families.

Panel Response

Since the issues presented in questions 7a-7d were overlapping, the Panel's response below addresses all parts of question 7. Historically, virus infected plants have been a part of the human and domestic animal food supply without adverse human or animal health effects. Exposure of PVC proteins generated in PVCP-PIPs that are identical with or within the range of natural variation of plant virus coat proteins would not alter the risk of toxicity and allergenicity.

Thus prior knowledge of the natural variation limits of the individual PVC proteins is required. The Panel expressed some disagreement as to whether the level of risk associated with human exposure to any protein is solely dependent on the protein itself. One Panel member concluded that the host producing the protein is of secondary importance. Others expressed concern related to expression of PVC-proteins in plants that are known to be highly allergenic such as peanut.

Truncated PVC proteins have been reported to occur in nature (Sacher and Ahlquist, 1989). Naturally occurring truncated forms of the PVCs could be generated by post-transcriptional and translational events, including incomplete translation due to routine errors causing a ribosome to dissociate from an mRNA, post-translational processing, the presence of a mutation that introduces a premature stop codon, or by infrequent translation initiation at downstream AUGs. Determining whether PVC-proteins containing terminal deletions, or any other modifications, are within the range of natural variation would require the development of a database of the natural variation and truncated forms of PVC-proteins that occur naturally. If a truncated PVC-protein does fall within the range of natural variation, the likelihood of increased toxicity and allergenicity would be low. Whether the truncation is at the N- or C-terminus is not relevant to allergenicity or toxicity.

Generally, the "free ends" are the least structurally constrained regions of a protein. As such, the ends can be thought of as being essentially "unstructured", and therefore unlikely to serve as allergenic epitopes or to make major contributions to the overall structure of the molecule. Addition (or deletion) of one or two amino acids is unlikely to change this. However, the possibility that the addition of amino acids such as cysteine with side chains that could promote cross-linking or aggregation between molecules should be considered. Beyond this, there are no general principles that can be used to determine how relative risk increases with an increasing number of added amino acids. Whether a methionine is added to the N- or C-terminus is not significant. The Panel appreciates why addition of a methionine at the N-terminus may be required. However, it was unaware of reasons why methionine would be added to the C-terminus. The addition of other amino acids containing reactive side chains that can serve as sites for post-translational modifications may be significant and should be evaluated on a case-by-case basis. The addition of more than one or two amino acids may be considered to be more than minimally modified.

Currently, it is extremely difficult to identify modifications that would be expected to be "within the range of natural variation for all virus families". This would require prior knowledge of the natural variation limits of the individual PVC proteins, which is not available. Specific modifications can be identified that would raise potential concerns, but it is not clear that it is possible to create a comprehensive list of these changes for all virus families. Such modifications might include the addition or removal of protease recognition sites, the addition or removal of cysteine residues involved in internal cross links, the addition or removal of proline residues that act as secondary structure "break points", and the addition or removal of asparagines and alanines involved in glycosylation. A description of the various amino acids that serve as targets for post-transcriptional modification can be found in Horton et al. (2005). Modifications such as single amino acid substitutions with biochemically similar amino acids that do not affect secondary or tertiary structure might be of relatively little concern. Given the

possible range of natural variations for PVC proteins, it would be appropriate to assess whether specific modifications are within natural variation limits of the PVC protein on a case-by-case basis.

Question 8

Please comment on the usefulness of the factors described above for evaluating food safety of the encoded PVC-protein. How important is it to characterize the expressed protein, e.g., to determine whether any post-translational modifications have occurred?

Panel Response

Most, if not all, food safety-related issues are addressed in the answers to the previous questions. The Panel suggested that the Agency consider the level of expression in determining whether to exempt a PVCP-PIP and believed that it would be difficult to elucidate a clear set of criteria to characterize "minimally modified" PVC-proteins.

Although the Agency did not specifically ask the Panel to comment on the issue, the Panel agreed that it appears appropriate to use naturally occurring PVC-proteins that are currently consumed in foods, and any available information on naturally occurring variants, as comparators for the "minimally modified" proteins. However, for both modified and unmodified proteins, the Agency might wish to consider adding a statement regarding expression levels to both paragraphs of criterion "c" as presented in the appendix of Attachment I of the Agency's background document "Draft Approach to Exempting Certain PVCP-PIPs from Regulation under FIFRA". This suggestion is based on the paragraph at the bottom of page 2 of Attachment II where the Agency states that "it anticipates that dietary exposure through … consumption of …residues of PVC-proteins …will be similar to or less than the dietary exposure to plant virus coat proteins currently found in food plants naturally infected with viruses." In this regard, it should be noted that most experts believe that exposure level is an important component of an allergenicity risk assessment. In addition, one Panel member indicated that low-level expression of PVC-proteins avoids induction of expression of pathogenesis related proteins, many of which are allergens.

Given the large number of potential modifications that might be made in a PVC-protein, it appears to be appropriate to evaluate each modification on a case-by-case basis. This includes both the determination that the protein is in fact "minimally modified" and whether the modified protein is "as safe as an unmodified protein." However, the Panel was not able to identify a clear set of criteria that can be used to define "minimally modified." Small changes in the sequence of a protein may have significant consequences for secondary and tertiary structure, as well as for the presence of a variety of important recognition sites. Examples of such sites can be found in Horton et al. (2005). It is critical to evaluate the protein as expressed in the host, including factors such as post-translational modifications. The Panel recognized the possibility that any modifications that are sufficient to move the protein out of "minimally modified" category would reduce or eliminate the efficacy of the construct. Further, as stated above and to the extent that

data are available, it is appropriate to compare modified PVC-proteins to the range of existing natural variants.

Similarly, constructs that "produce no protein" describe a situation with minimal food safety risk. However, it might be appropriate to consider distinguishing situations in which the construct used can never produce protein from one where no protein is produced from a functional gene construct.

Question 9

9(a). **What is the likelihood that a chimeric PVC-protein would have increased toxicity or allergenicity relative to the corresponding non-chimeric plant virus coat proteins? Can you describe any objective criteria to identify those chimeric PVC-proteins with novel toxic or allergenic properties?**

9(b). **Please address the relevance of the following factors to the potential toxicity or allergenicity of a chimeric PVC-protein:**

(i) the size of the various segments comprising the chimeric PVC-protein,
(ii) the viral source(s) of the various segments, and/or
(iii) the location on the protein where fusions occur.

9(c). **Are the factors specified in question 8 applicable to evaluating the safety of chimeric PVC-proteins? Are there any additional factors specific to chimeric proteins that should be considered?**

Panel Response

Since the issues presented in Questions 9a-9c are overlapping, the Panel's discussion below addresses all parts of Question 9.

The Panel was not clear on the Agency's definition of a chimeric protein. A chimeric protein is the fusion between two (or more) capsid proteins. The Panel concluded that a chimeric protein, under this specific definition, would constitute a completely novel protein and should be considered as such. The rationale behind this conclusion was that each one of the fused capsid proteins would be too different from a minimally modified one. Unless the two capsid proteins are separated post- or co-translationally, e.g., proteolytically due to an engineered protease recognition site, they cannot be considered minimally modified. The concern was that a new, chimeric protein would have completely different antigenic and possibly allergenic properties compared to the properties of the individual capsid proteins. Multimerization or aggregation to the extent beyond what is normally characteristic of the original individual CPs was also a concern. Emphasis should be placed not on food-mediated exposure, but on exposure through inhaled material.

The Panel indicated that it may be difficult to make specific predictions as far as increased toxicity and/or allergenicity relative to the original CPs constituting the chimeric

protein (Breiteneder and Mills 2005). Production level may be the most important parameter; however, ability to form multimers or aggregates that differ from those formed by the original CPs, is an undesirable feature of the chimeric protein.

Extra sequences should be taken into account. These include: 1) N-terminal methionine and signal/leader sequences, 2) targeting signals, 3) ER-retention signals, and; 4) tagging sequences. All of these extra sequences should be considered separately.

1) N-terminal methionine and signal/leader sequences. These are not a great concern. They are removed when a nascent peptide chain is 20-30 amino acid residues in length (Moerschell et al., 1990; Bradshaw et al., 1998; Lowther et al., 2000).

2) Targeting signals, which guide the protein to a certain organelle/compartment membrane, are not removed. These need to be addressed, but discussants did not have any examples of enhanced toxicity/allergenicity. They may require regulation on a case by case basis.

3) ER-retention signals, which arrest the protein in the ER, are not removed. Since plant-specific N-glycosylation occurs in the Golgi complex (Faye et al. 2005), i.e., downstream of the ER, and is known to cause immune response to plant-specific types of glycans (Bardor et al. 2003), the presence of ER-retention signals in plant-produced proteins may be considered beneficial (Ko et al., 2003; Tekoah et al., 2004). With ER-retention signal present, N-glycosylation sites need not be removed. Conversely, deletion of a known ER-retention signal may result in a protein with plant glycans attached that may cause immune or allergic reaction to the carbohydrate parts of such a protein. Thus, deletion of the ER-retention signal may need to be augmented with the removal of the N-glycosylation sites as well. Presence or absence of N-linked glycosylation sites is important unless expression occurs in chloroplasts. Moreover, glycosylation may modify the tertiary structure of the overall protein creating new epitopes or shielding/removing existing epitopes compared to the original, wild-type protein. O-glycosylation is important in allergenicity. However, signals of O-glycosylation are less defined, and modification itself is less understood.

4) Tagging sequences, introduced for tracking or purification purposes, are not removed. Popular tags are His-6, FLAG, Myc, etc. These tags are fused to the protein for tracking purposes, are not removed, and generally are considered safe, but may require separate clearance through FDA.

Question 10

Please comment on the Agency's environmental risk assessment of each of the six selectable markers (found in attachment III). Does the SAP concur that CP4 EPSPS, GOX/GOXv247, PAT each pose a low probability of risk to the environment when used in one of the plants listed in question 1(a)? Does the SAP concur that beta-D-glucuronidase, NPTII, and PMI each pose a low probability of risk to the environment when used in any plant?

Panel Response

In preparing its response to this question, the Panel decided that the selectable marker genes under consideration fell into three groups: herbicide resistance, antibiotic resistance and other. The antibiotic resistance marker (NPTII) and other markers (GUS and PMI) should be exempt provided they were in the plant species determined to be of low risk using criteria listed in Question 1. The herbicide markers (CP4 EPSPS, GOX/GOXv247 and PAT) should not be exempted, but rather should be considered on a case-by-case basis taking into consideration the potential that the crop plant has to become feral. The Panel came to this decision based on the knowledge that glyphosate and glufosinate are among the two most common herbicides used to eradicate feral species from non-agricultural ecosystems and volunteers within agroecosystems. Having these in PVCP-PIP species would preclude the use of this most important tool, should an exempted species, against all best wisdom, become feral or become a volunteer weed in agroecosystems. Because these two herbicides are among the most environmentally innocuous, their use is to be preferred in all ecosystems.

Selectable marker genes are incorporated into constructs and inserted into plants to select those plants that have been successfully transformed. As they are expressed, they have to be considered alongside the main trait that has been introduced into the plant, which, in this instance, is PVCP-PIP. The issue of environment was an important consideration of the Panel. Three environments were identified by the Panel: 1) the agronomic environment comprising arable, pasture, forest, fish farm, etc., in which there is continuous application of inputs; 2) the peri-agricultural environment, which comprises the edges of the agronomic environment in which there would be some intentional application of pesticides and also flow of inputs from the agronomic environment; and 3) the natural environment in which there would be no pesticide inputs. Although it was recognized that there would be some interactions among these environments, it was considered that the comments relating to this question should apply to the natural environment.

The Panel also provided specific comments on the six selectable markers. The comments are provided below.

CP4 enolpyruvylshikimate-3-phosphate (CP4 ESPS)

This marker confers tolerance to the herbicide, glyphosate. The Panel identified several references indicating that this marker has been considered acceptable in the US and EU. In 1996, EPA established an exemption from the requirement of a tolerance for residue (EPA 1996). The European Union Scientific Committee on Plants (European Union 1998a) gave an opinion that “there is no evidence indicating that the use of fodder beet tolerant to glyphosate with the purpose to be used as any other fodder beet is likely to cause any adverse effects on human health and the environment."

Other research on CP4 ESPS was conducted by Sten et al. (2004), reporting no allergenic problems associated with soybean containing CP4 ESPS. Rats and poultry fed with CP4 ESPS corn showed no difference from those fed with non-GM corn (Hammond et al., 2004; Taylor et

al., 2005) demonstrating that any detrimental effects to mammals and birds in the natural environment would be unlikely.

Glyphosphate oxidoreductase + version 247 (GOX/GOXv247)

GOX/GOXv247 confers tolerance to glyphosate. As with CP4 ESPS tolerance, tolerance to GOX/GOXv247 has been considered acceptable both in the US and European Union. As an example, EPA established an exemption from the requirement of a tolerance for residues of GOX and GOXv247 (EPA 1997).

Phosphinothricin acetyltransferase (PAT)

PAT confers tolerance to the herbicide glufosinate (glutamine synthetase). The European Union Scientific Committee on Plants (September 5, 2001) stated "no evidence to indicate that the use of the genetically modified maize [containing PAT], as any other maize, is likely to cause adverse effects on human or animal health and the environment" (European Union 2001).

β-D-glucuronidase (GUS)

GUS gives a color reaction. Both the Canadian Food Inspection Agency (Canadian Food Inspection Agency 2003) and an EPA Biotechnology Consultation (EPA 2002) concluded that there was no evidence for allergenicity or toxicity in Bolgard cotton containing the GUS gene.

Neomycin phosphotransferase II (NPTII)

NPTII confers resistance to aminoglycoside antibiotics, e.g., kanamycin and neomycin. In a recent review, Gay and Gillespie (2005) stated "We conclude that, although fragments of DNA large enough to contain an antibiotic-resistance gene may survive in the environment, the barriers to transfer, incorporation and transmission are so substantial that any contribution to antibiotic resistance made by GM plants must be overwhelmed by the contribution made by antibiotic prescription in clinical practice." Furthermore, the European Union Scientific Committee on Plants (1998) "considers that there is no evidence indicating that the seeds of AgrEvo glufosinate ammonium tolerant genetically modified oilseed rape [containing the NPTII marker gene], to be imported and processed in the manner indicated, are likely to cause adverse effects on human or animal health and the environment."

Phosphomannose isomerase (PMI)

PMI catalyzes the inter-conversion of mannose-6-phosphate and fructose-6-phosphate. Plant cells lack the enzyme and are incapable of surviving on synthetic media containing mannose as a carbon source. There was no detectable glycoprotein changes in maize, wheat, barley or watermelon containing PMI (Reed et al., 2001), no evidence of allergenicity in PMI-containing soybean (Sten et al., 2004) or any effects on rats fed on corn containing PMI (Hammond et al., 2004).

The Panel also provided some general overall comments about selectable markers and PVCP-PIPs.

As the risk may depend on gene flow, risk containment and risk mitigation, considerations should be employed for the use of markers in a crop plant. There is a need to define high risk crops and the agricultural practices associated with the use of these crops, and monitor them on a case by case basis, with a focus on research questions regarding safety and risk to these crops and subsequent evaluation of risk mitigation/containment mechanisms.

The Panel debated whether there was a greater potential for protein expression to be observed in plant species with a shorter life cycle. The discussion ranged from views that the current Agency risk assessment has not addressed this issue, to views that it was not a significant problem.

Question 11 - Criteria and Evaluation of Other PIPs Conferring Virus Resistance

Panel Summary

Other PIPs conferring virus resistance should be evaluated similarly as are the PVCP-PIPs, if the PIPs mode of action is via PTGS. Only a few virus genes, which if designed to express proteins in the transgenic plants, should be evaluated separately. These are genes encoding proteins that are silencing suppressors and those involved in virus cell-to-cell movement.

Please comment on whether the criteria discussed above that EPA is considering for PVCP-PIPs (i.e., relating to gene flow, viral interactions, and protein production) would be applicable for other PIPs conferring virus resistance, e.g., those based on virus replicase genes (Ehrenfeld et al. 2004) or defective interfering RNA (Kollar et al. 1993). Please indicate the scientific rationale for including any additional PIPs under such an exemption and whether any additional (or fewer) qualifications would be needed.

Panel Response

Other PIPs conferring virus resistance should be evaluated similarly as are the PVCP-PIPs, if the PIPs mode of action is via PTGS. Scientific rationale supporting this includes that we already have extensive agricultural experience with PVCP-PIPs, particularly those in squash and papayas, and the use of other types of resistance genes without detectable novel or negative effects (Gonsalves, 1998; Tricoli et al., 1995; Lin et al., 2003; Fermin et al., 2005). This has to be considered in future exemption considerations. It is important to build from the positive effects resulting from using those genes in U.S. and worldwide agriculture, and not only the potential negative effects.

However, the Panel mentioned several phenotypes that are associated with specific virus proteins and suggested that perhaps these proteins should not be exempt if the construct used in the transgenic plant is designed to express these proteins. Two important examples are virus proteins that suppress PTGS and those that are known to facilitate virus cell to cell movement.

Each could potentially affect the phenotype in the transgenic plant if it were to be infected by a virus other than that which it is designed to protect against. Proteins that facilitate virus replication, and even vector transmission, while theoretically problematic, likely present a very low probability for the production of novel phenotypic effects in transgenic plants. The Panel believed that it was unlikely that these types of constructs will be used based on today's knowledge. Again it was stated that approaches today are almost all PTGS-based. These are recognized today as the most effective, and it makes no difference which virus genomic regions are used if the approach is to confer resistance via PTGS. If these are designed so as to confer virus resistance, then gene flow issues also are no different than were those for PVCP-PIPs. The human health related issues related to any protein are the same as elucidated for the PVCP-PIPs, as are the problems. Knowledge of previous exposures and the nature of natural variation serve as the appropriate comparators for any risk assessment.

The Panel also provided overall comments in response to the Agency's position papers and the 1992 FIFRA SAP reviewing PVCP-PIPS. Stating that expression levels of other virus genes/products (other than CPs) in transgenic plants engineered for virus resistance is fundamentally different than for CPs is incorrect. This statement suggests that CPs are the most highly expressed proteins in virus infections, and expressing virus proteins other than virus CPs in transgenic plants will give higher levels of these proteins than are found naturally in virus infections, and therefore may increase potential for interactions. This is not a valid assumption. Many viruses, for example those of the *Potyviridae* (picornavirus lineage) do express all gene products (proteins) essentially equally. The 1992 Panel also discussed protein turnover, and evidence for protein turnover may not be correct, and in many cases may not have been investigated. Thus, this cannot be used as a criterion without more information. Certainly for potyviruses, many of the other gene products (proteins) in fact do not turn over; instead they accumulate as distinct inclusion bodies composed of the virus-encoded proteins. Thus, it is important to clarify that expression of other virus genes, versus those for coat proteins, may not be different from an expression or accumulation perspective.

In addition, on a per cell basis, it is almost certain that all viral gene products will be expressed higher in a virus infection than they will be in transgenic plants. If frequency of product per unit of space and time affects probability for interaction, then probability for interaction is likely to be greater in a virus-infected plant than in a transgenic plant.

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