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WASHINGTON D.C., 20460

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

May 27, 2009

MEMORANDUM

SUBJECT: Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting Held February 25 - 26, 2009 on the Data Required to Register Plant-Incorporated Protectants

TO: Debbie Edwards, Ph. D.
Director
Office of Pesticide Programs

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

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

Frank Sanders, Director
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 February 25 - 26, 2009. This report addresses a set of scientific issues being considered by the Environmental Protection Agency pertaining to the Data Required to Register Plant-Incorporated Protectants.

Attachment

cc:

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OPP Docket

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SAP Minutes No. 2009-04

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

The Data Required to Register Plant-Incorporated Protectants

February 25 – 26, 2009
FIFRA Scientific Advisory Panel Meeting
held at the Environmental Protection Agency
Conference Center
Arlington, VA

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). The meeting minutes represent the views and recommendations of the FIFRA SAP, not the United States Environmental Protection Agency (Agency). The content of the meeting minutes does not represent information approved or disseminated by the Agency. The meeting minutes have not been reviewed for approval by the Agency and, hence, the contents of these meeting minutes 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 is a Federal advisory committee operating in accordance with the Federal Advisory Committee Act and established under the provisions of FIFRA as amended by the Food Quality Protection Act (FQPA) of 1996. The FIFRA SAP provides 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 Environmental Protection Agency, Office of Pesticide Programs (OPP), and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. FQPA Science Review Board members serve the FIFRA SAP on an ad hoc basis to assist in reviews conducted by the FIFRA SAP. Further information about FIFRA SAP reports and activities can be obtained from its website at http://www.epa.gov/scipoly/sap/ or the OPP Docket at (703) 305-5805. Interested persons are invited to contact Joseph E. Bailey, SAP Designated Federal Official, via e-mail at bailey.joseph@epa.gov.

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

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February 25 – 26, 2009
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Steven G. Heeringa, Ph.D.
FIFRA SAP Chair
FIFRA Scientific Advisory Panel
Date: May 27, 2009

Joseph E. Bailey
Designated Federal Official
FIFRA Scientific Advisory Panel
Date: May 27, 2009

Federal Insecticide, Fungicide and Rodenticide Act
Scientific Advisory Panel Meeting
February 25 – 26, 2009

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Kristin L. Mercer, Ph.D., Assistant Professor, The Ohio State University, Department of Horticulture & Crop Science, Columbus, OH

INTRODUCTION

The Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP) has completed its review of a set of scientific issues being considered by the Agency that are associated with the data required to register Plant-Incorporated Protectants (PIP). Advance notice of the meeting was published in the *Federal Register* on December 10, 2008. The review was conducted in an open Panel meeting held in Arlington, Virginia, from February 25 – 26, 2009. Dr. Steven G. Heeringa chaired the meeting. Joseph E. Bailey served as the Designated Federal Official.

This Scientific Advisory Panel meeting addressed selected scientific issues associated with the data required to register plant-incorporated protectants (PIPs). A PIP is a pesticidal substance that is intended to be produced and used in a living plant, or in the produce thereof, and the genetic material necessary for production of such a pesticidal substance. The term includes both active and inert ingredients. PIPs are regulated as pesticides by the Environmental Protection Agency (EPA) under FIFRA because they meet the FIFRA definition of a pesticide, being intended for preventing, destroying, repelling, or mitigating a pest. EPA sought the assistance of the FIFRA SAP to evaluate several scientific issues associated with the data required to support registration of PIPs including gene/protein nomenclature, bioinformatics assessment of novel proteins, synergistic effects of multiple PIPs in a plant, soil microbial community effects, and the environmental assessment of gene flow. The SAP review, along with other past PIP-related SAP reviews, will be used by the Agency to assist in preparing a proposed rule to establish data requirements for pesticides classified as PIPs. This rule would propose to codify the data requirements to regulate experimental use permits and register PIPs, thereby improving the Agency's ability to make regulatory decisions about human health and environmental effects of these pesticide products.

Steven Bradbury, Ph.D., (Deputy Office Director for Programs, Office of Pesticide Programs) and Janet Andersen, Ph.D., (Director, Biopesticides and Pollution Prevention Division) provided opening remarks at the meeting. The agenda for the meeting included public comments and Agency presentations by Chris Wozniak, Ph.D., Annabel Waggoner, and Zig Vaituzis, Ph.D. of the Biopesticides and Pollution Prevention Division, Office of Pesticide Programs.

PUBLIC COMMENTS

Oral Statements were presented as follows:

Richard E. Goodman, Ph.D., University of Nebraska-Lincoln
Keri Henderson, Ph.D., on behalf of Agricultural Biotech Stewardship Technical Committee
Rod Herman, M.S., on behalf of Agricultural Biotech Stewardship Technical Committee
Greg Ladics, Ph.D., on behalf of The Protein Allergenicity Technical Committee, International Life Sciences Institute, Health and Environmental Sciences Institute
Raymond Layton, Ph.D., on behalf of Agricultural Biotech Stewardship Technical Committee
Steven Levine, Ph.D., Michael Horak, Ph.D., and David Carson, Ph.D., on behalf of Monsanto Company
Laura Privalle, Ph.D., on behalf of Croplife International Expert Team on Allergy
Steven Strauss, Ph.D., Oregon State University
Demetra Vlaclos, Syngenta Seeds, Inc.

Written Statements were provided by:

Denise Dewar, on behalf of CropLife International
Nancy G. Doerrer, M.S., on behalf of International Life Sciences Institute, Health and Environmental Sciences Institute, Protein Allergenicity Technical Committee
Richard E. Goodman, Ph.D., University of Nebraska-Lincoln
James Hancock, Michigan State University
Karen E. Hokanson, Ph.D., (University of Minnesota), Hector Quemada, Ph.D., (Calvin College), and Rebecca Grumet, Ph.D., (Michigan State University) on behalf of Program for Biosafety Systems
Rachel Lattimore on behalf of the Agricultural Biotechnology Stewardship Technical Committee
Steven Levine, Ph.D., David Carson, Ph.D., Andre Silvanovich, Ph.D., and Keith Reding, Ph.D.,on behalf of Monsanto Company
Les Pearson, on behalf of ArborGen LLC
William A. Powell, Ph.D., on behalf of the American Chestnut Research and Restoration Project, SUNY College of Environmental Science and Forestry
Charles Maynard, Ph.D., on behalf of the American Chestnut Research and Restoration Project, SUNY College of Environmental Science and Forestry
E. Shields, et. al.
Jeffrey D. Wolt, Ph.D., Iowa State University of Science and Technology
Elson Shields, Ph.D., Cornell University
R. White
Michael Wach on behalf of Biotechnology Industry Organization

SUMMARY OF PANEL DISCUSSIONS AND RECOMMENDATIONS

The FIFRA Scientific Advisory Panel (SAP) met on February 25 - 26, 2009 at the Environmental Protection Agency's Potomac Yard Conference Center to listen to public comments and Agency presentations and to discuss their recommendations on four sets of charge questions concerning the data required to register plant incorporated protectants (PIPs).

Charge Question A.l concerned the nomenclature for PIPs, particularly the naming of proteins and RNAi's. The goal of the EPA in considering the nomenclature of PIP products is to provide a description for each PIP product "such that the public can understand what the product is and how it functions as a pesticide." In general, the Panel agreed with the EPA's current draft document on nomenclature for proteins, which they described as "Protein designation + source + effect." The situation for RNAi's is more complex because there is no standard nomenclature within the scientific community for this class of gene products. The Panel suggested use of a variant of the nomenclatural format for proteins, with the format depending on whether the target was a protein expressed in the host or one expressed in a virus or pathogenic organism. In addition to their nomenclatural recommendations the Panel suggested that it would be very desirable to make full use of publicly available databases to link PIP specifications to other publicly available knowledge. Finally the Panel suggested that a full technical terminology that goes beyond common names for PIP products, e.g., gene and protein sequences or enzyme classification (if available), might be needed in the future.

Charge Question B.1 requested that the Panel comment on whether the current six to eight amino acid epitope search provides a statistically significant and biologically meaningful approach for addressing allergenicity risk in the absence of the 35 percent identity trigger? The Panel was also requested to provide recommendations on criteria for judging databases for their validity and completeness to address allergen, toxin, anti-nutrient and other hazardous protein similarities.

With respect to the six to eight epitope search, the Panel acknowledged that some details of the rules are not optimal; however, the Panel strongly felt that both rules appear to have worked well in the past and that they effectively serve the purpose of flagging situations where additional allergenicity testing is required. The number of situations that have required further examination as a result of the use of the criteria is quite small (only two documented cases), and thus the criteria do not appear to represent an extraordinary burden for PIP registrants. There was a general consensus among the Panel members that it would be premature to modify the six to eight amino acid similarity criterion or to completely abandon the 35% identity trigger.

Use of databases for their validity and completeness concerning allergen, toxin, anti-nutrient and other hazardous protein similarities was judged to be problematic. The Panel believed that there are no overarching principles that can be used to say that one

database's approach is valid and another is not, or that one of the databases is complete while another is not. The "completeness" of an allergen database is a vague concept that cannot be tested. For each available database, criteria for its contents and structure were used that the developer felt were appropriate for the intended use of that database. Rather than attempt to define the "perfect" database and a static set of standards for making judgments, the Panel suggested that it would be more useful to generate empirical data on the consequences of using different data sets with a number of different query sequences.

Charge Question C.1 asked the Panel to comment on methodology to support the analysis for synergistic effects for combination PIP products and any additional comments on this approach, including identifying instances where the Agency would be justified in requiring additional testing with a combination test substance containing a mixture of two or more active ingredients for data development on human health and non-target organism effects.

In general, the Panel believed that studies showed that synergism is not easily predicted, and therefore testing for synergistic effects when two or more PIPs are combined is warranted. In cases where the mode of action of two PIPs is known and they are similar, the testing for synergism using a protocol similar to that outlined by EPA would be appropriate for lepidopteran and coleopteran insects. However, where multiple PIPs that have different modes of action are being combined, the protocols would have to be tailored to test the combinations, taking into account their different modes of action. In cases where PIPs with different, and especially novel modes of action are combined, the Agency would be justified in requiring additional testing for human health and non-target organism effects.

Charge Question C.2 asked the Panel to comment on whether testing the microbial populations in soil ecosystems should be limited to examining effects on activity of beneficial soil microbes, specifically carbon (C) and nitrogen (N) cycling at the soil microbial community level. In general, the Panel pointed out that testing only for C and N transformations may not reflect actual changes to the microbial community composition, which could be important. Respiration assays assess very broad functional activity and alterations in sensitive populations could be masked. The Panel supported the selection of ammonification as a critical test because it focused on a selective group or segment of the soil microbial community, although other subgroups could be more sensitive to the PIP. Nitrification is a good assay for monitoring as a component of the total N cycle, but basing this on the end product, NO_3, is problematic. Soils vary widely in nitrifier populations and it may be more useful to consider nitrifier detection instead of measures of nitrate levels. The Panel suggested considering a multiphasic approach as a more comprehensive analysis of microbial community structures and function in response to PIP exposure, especially if initial microbial assays revealed complex and varied responses. The Panel also suggested the molecular analysis that targets functional genes, such as those that target nitrification or denitrification, could be used to indicate soil function and the diversity of the group of organisms responsible for those functions. Because of the dynamics of soil microbiology, attempting to duplicate soil conditions and microbial communities in the laboratory is extremely difficult.

Charge Question D.1 was broken into four subsections, a-d. All four concerned assessment of whether particular types of data would be informative concerning the effects of gene flow of a PIP from a crop to a sexually compatible wild relative (SCWR). The specifics of the data to be assessed and the potential risk or harm varied among the four subsections, but in all cases, the Panel's judgment concerning the value of the data was tempered by studies from ecology and evolution demonstrating that effects on organisms and ecological communities are often very complex and vary spatially and temporally due to variable genetics of the SCWR and the species with which it interacts, as well as other biotic and abiotic factors that affect a species' population dynamics and competitiveness.

Subsection D.1a asked the Panel to discuss whether it is possible to evaluate, in part, impacts of a gene flow event by gathering data on target (pest) species which are associated with the wild species (transgene recipient).

The potential impacts of gene flow from a crop to a SCWR are many, ranging from direct effects on the ecology and evolution of the SCWR, to secondary ecological and evolutionary effects on the target and non-target species directly affected by the presence of the PIP in the SCWR, to broader community ecological effects. For this reason, the Panel believed that, although it might be possible to glean some information on impacts of gene flow from data on the target species associated with the SCWR, these data would only be of very limited value in assessing the overall potential impacts of gene flow. Although not specifically requested by the EPA, the Panel made suggestions concerning other data that could be gathered to better address impacts of gene flow.

Subsection D.1b asked the Panel to discuss whether the data gathered on target species will allow estimating the degree to which resistance to these target species may influence the population dynamics or invasiveness of the wild relative.

As with subsection D.1a, the Panel judged that data gathered on the target species could only be of very limited value in determining whether PIP-produced resistance in the SCWR would influence the population dynamics or invasiveness of the SCWR. Altered population dynamics and invasiveness are complex features of a population that depend on more than whether the target species is impacted by a PIP that has introgressed into a SCWR. Here again, the Panel provided suggestions on data and experiments that would be better suited to assessing altered population dynamics or invasiveness.

Subsection D.1c asked the Panel to discuss whether empirical data regarding the target species (e.g., fungi, insects, etc.) and non-target species (e.g., pollinators, detritivores) associated with the sexually compatible wild relative have the potential to inform about risks to the SCWR population and the associated community.

The Panel judged that data from target and non-target species associated with the SCWR could have the potential to inform about risks to the SCWR and the associated community, but only to a limited extent. The reasons the data from these species is only

of limited value springs from the same set of issues that limit the value of the data considered for D.1a and D.1b. Again, the Panel suggested other data and experiments that would more directly address the concern of the subsection.

Subsection D.1d asked the Panel to discuss whether an understanding of the potential effect(s) of introgressed transgenes on basic plant habit, phenology and physiology provide a basis for assessing potential impacts following a gene flow event.

The Panel believed that the same kind of studies described for subsections D.1a-c would be necessary for exploring effects on fitness, population dynamics, and competition.

The effects of a PIP that influences habit would depend crucially on the same factors identified in D.1a: the expression of the PIP in the SCWR, any interactions with other alleles controlling habit already present in the SCWR, and the importance of the PIP characteristic in determining the SCWR's fitness. Knowledge of changes in the SCWR's phenology due to expression of the PIP could be useful in assessing the effects of gene flow, but by itself would not likely be sufficient to fully understand gene flow's impacts on the SCWR and the ecological community. Phenological changes could alter gene flow potential (crop-wild and wild-wild) or alter characteristics that are important determinants of fitness. Altered physiology covers a vast range of potential effects on the SCWR, and, therefore, its interactions with other members of the ecological community. It is difficult to say with any precision whether knowledge of physiological changes would aid in assessing impacts of gene flow.

Concerns were expressed by one Panel member that some of the guidelines, especially those related to gene flow between PIP plants and sexually compatible wild relatives, could impede and potentially stop the production of PIPs of great ecological and/or economic importance. An example of a plant that could benefit from PIP technology is the American Chestnut, *Castanea dentate*, once a common tree in Eastern North American hardwood forests that has been devastated by Asian bark fungus, *Cryphonectria parasitica* (a.k.a chestnut blight). The Panel member recommended that the EPA should provide avenues for discussion of such PIPs for interested scientists so that on a case-by-case basis risk/benefit assessments could be made along with appropriate problem formulation to identify concerns, uncertainties and an analytical plan to address needs.

PANEL DELIBERATIONS AND RESPONSE TO CHARGE

Charge Question A.1 – Please comment on the Agency's approach to identification of proteins found in PIPs to describe the active ingredient and its function as a pesticide. Please comment on the use of the gene name or phenotype in PIPs developed using RNAi. Is there another more systematized naming system that could be employed for either proteins or RNAi? Please provide a basis for your answer.

Panel Response - Description of the active ingredient of a PIP: The charge of the Panel is restricted to the nomenclature describing novel PIP products, however, the Panel believed that the impact of the nomenclature should also be considered in the context of risk assessment. In that context, the proposed nomenclature should allow an expert in the field as well as an informed lay person to identify the novel PIP product as a molecular entity as precisely as possible. In addition, it should enable EPA personnel and the public to find relevant information on that product in publicly available databases (see Appendix A).

The goal of the EPA in considering the nomenclature of PIP products is to provide a description for each PIP product "such that the public can understand what the product is and how it functions as a pesticide." The Agency also refers to this as providing a "common name for the PIP." In other words, the Agency wishes to derive common names from technical information about the PIP products. This presents a challenge, as numerous or ambiguous common names must be mapped to a specific technical terminology. The examples given by the Agency implicitly assume that the common names should be based on the identity of the expressed protein, when there is one. Further, the Agency specifically asserts that the common name should include information on the intended function of the PIP. The Panel agreed with these assumptions and followed the suggestions as given in the position paper to a large extent, with the following specific modifications.

The basic format for nomenclature of a PIP product can be described as "Protein designation + source + effect." One Panel member suggested that a database accession number could be included as part of the designation if it were available or if it were needed to specify a specific variant or allele.

In the simplest case, the PIP construct of a plant expresses an unmodified protein, i.e., a protein that is identical to a natively occurring protein in another organism. In that case the generally accepted protein name along with the intended effect appears appropriate. The example given in the position paper as a "Cry2Ba protein from *Bacillus thuringiensis* for control of lepidopteron pests" would follow the basic format.

For modified PIP proteins, the basic format can be retained if the protein designation contains a common name such as "A-like" for naturally occurring protein A. The nomenclature suggested covers most cases where a natural protein product has been extensively modified to achieve the pesticidal function. One Panel member also suggested use of the designation of a protein family according to a generally accepted

protein classification such as Pfam (Finn et al., 2008). Pfam (Pfam A) is a comprehensive collection of protein domains and families, manually curated and supported by state-of-the-art bioinformatics software tools. Pfam covers most currently known protein sequences in its current release (22.0) with 9318 protein families. Pfam can be searched by sequence to find the family of most closely related proteins of a novel PIP product. The annotation in Pfam is based on individual domains and can thus also be used if chimeric protein constructs are part of the PIP.

Use of the gene name or phenotype in PIPs developed using RNAi: Silencing genes through RNA interference (RNAi) technology is a recent development in genetic research and biotechnology with a broad range of applications, including pest control. RNA interference (RNAi) is the specific downregulation of gene expression by double-stranded RNA (dsRNA), where the sequence of one strand of the dsRNA is identical to parts or all of a specific gene transcript. The downregulation of the target gene is sequence specific and mediated by degradation of the target mRNA. The degradation is initiated through the production of small interfering RNAs (siRNAs) from the dsRNA, which is cleaved by dsRNA-specific endonucleases referred to as dicers.

RNAi technology extends the previous use of transgenic plants, e.g., by expressing the Bt gene, in pest control. Recently two studies demonstrated the feasibility of the approach by feeding insect larvae with the plant material expressing dsRNA for targeted insect genes (Baum et al., 2007, Mao et al. (2007). The key to the success of this approach is (i) identification of a suitable insect target and (ii) dsRNA delivery, which includes *in planta* expression of dsRNA and delivery of sufficient amounts of intact dsRNA for uptake by the insect. In both cases the RNAi pathway in insect larvae blocked the expression of the target genes.

RNAi technology is rapidly evolving, and there is no universally accepted database. However, there are several databases where critical information, such as siRNA sequence and gene location of the target gene, can be required as a precondition for registration and information for the public.

No uniform nomenclature is currently available for RNAi products. Researchers in the field use featureless labels such as "line 1" and "line 2" or base the designation on a hopelessly obscure description of the genetic construct used in the development of the PIP. The best approach would be to use a variant of the nomenclatural format described above, with the format depending on whether the target was a protein expressed in the host or one expressed in a virus or pathogenic organism. In the first case, the PIP could be described as, for example, "*receptor-minus* for fungal resistance" (using the proper name of the receptor and fungus as appropriate). When the silencing is aimed at an external pathogen, the format could be "*coat protein* silencing for virus resistance" (again using proper names as appropriate). As a tool for transmitting information to the public, the Panel did not believe that it is critical to worry about the technical uses of terms such as silencing, inhibition, suppression, etc. However, using consistent terminology and structure is important.

In summary, the Panel followed closely the suggestion of the Agency, and suggested making full use of publicly available databases to link PIP specification to other publicly available knowledge. Finally the Panel suggested that a full technical terminology that goes beyond common names for PIP products, e.g., gene and protein sequences and enzyme classification (if available) might be needed in the future.

Charge Question B.1 – Given the negative results of specific serum screening of novel proteins triggered by identification of six to eight segment searches for allergenic epitopes to date, please comment on whether the six to eight amino acid epitope search provides a statistically significant and biologically meaningful approach for addressing allergenicity risk in the absence of the 35 percent identity trigger? Please provide recommendations on criteria for judging databases for their validity and completeness to address allergen, toxin, anti-nutrient and other hazardous protein similarities. Please provide a basis for your answer.

Panel Response - In order to fully answer this question, the Panel believed that it was important to consider the context in which bioinformatics assessments of novel proteins are used. As stated in the Agency Position Paper, bioinformatics is used as part of a "weight of evidence" approach for assessing human health risks. The human health risk that has received the most attention is that of allergenicity.

The need to apply a "weight of evidence" approach to allergenicity assessment is the result of the fact that there are no known tests, indicators, or protein properties that can be considered definitively predictive of allergenicity. This situation reflects extensive and far reaching lack of information on the biology of food allergy. Scientists do not know why some people develop allergies, and others do not; why some foods are more likely to be allergenic that others; or why sensitive individuals develop antibodies to different arrays of proteins in a single food. It is not even known whether the development of an allergy is best thought of as a positive process (active development of sensitivity) or a negative process (failure of oral tolerance) or a combination of the two.

In the face of this extensive information gap, the best approach to assessing the potential allergenicity of a novel food protein has been to ask a number of questions about that protein. The Panel noted that history suggests that there would be considerable value in considering how to improve our use of all the answers to these questions in the "weight of evidence" approach. However, the Agency did not ask the Panel to consider that question.

Although bioinformatics has always been an important component of allergenicity assessment, the Panel noted that the role of bioinformatics should be specifically defined as "raising potential red flags" on a potential allergen rather than an actual prediction of allergenicity. Bioinformatics is a screening tool that can be used to identify situations potentially requiring further analysis. One Panel member pointed out that saying the results of any particular analysis are "false positives" or "true negatives" is incorrect and misleading. An analysis that finds a match or similarity between two protein sequences is giving real positives for further analysis as long as the sequences do, in fact, match. It

is not an indication that any one of the regions is a true negative or false positive. It simply is a measure of the number of candidates for further analysis. The number of such matches and the need for further consideration of these matches are not in themselves indications of relevance or irrelevance.

Bioinformatic analyses related to potential allergenicity are carried out by comparing the amino acid sequence of a query protein to a database containing sequences of known food allergens. The Panel agreed that the EPA has correctly recognized that the contents of the database (or databases) used is a critical consideration.

The diversity in the existing allergen sequence databases reflects differences in the criteria used to construct these databases. This diversity has been described in Gendel (2009) and Gendel & Jenkins (2006). In each case, criteria were used that the developer felt were appropriate for the intended use of that database. The Panel believed that there are no overarching principles that can be used to say that one approach is valid and another is not, or that one of the resulting databases is complete while another is not. In addition, the "completeness" of an allergen database is a vague concept that cannot be tested. Rather than attempt to define the "perfect" database and a static set of standards for making judgments, the Panel suggested that it would be more useful to generate empirical data on the consequences of using these different data sets with a number of different query sequences. Further, one Panel member observed that the diversity of current allergen databases could be an advantage in the application of the FAO/WHO criteria. Nevertheless, the Panel suggested that a number of factors can be used to characterize each database and that those described in the draft guidelines are a good start.

A full characterization would include the following: (1) a complete description of the sequence sources, (2) the criteria used to include and exclude sequences, (3) whether any sequences were altered such as by removal of leader or transit sequences, and (4) how isoallergens and sequence variants are treated.

If duplicate sequences are removed from the database, the definition of "duplicate sequences" that was used should be provided. It was suggested that full characterization of a database should be defined as providing sufficient information to allow another scientist to recreate the same database. Thus, for example, if considerations such as expert opinions are used, the reasoning for each decision (both positive and negative) made by the experts should be made accessible. The Panel also recognized that sequence databases change over time. Therefore, they suggested that it was important for the agency to have a complete description of the actual contents of a database at the time that it was used to generate data contained in a submission. This could be in the form of a version or release number, as long as that version or release is available.

The Panel noted that the Agency's draft guidelines point out that use of publicly available databases is preferred. In this context, the Panel suggested that it is important to clarify exactly what is meant by "publicly available." As used in the draft guideline, it appears that the Agency intends for this term to refer to repository databases such as

GenBank or UniProt. However, a "derived" database containing allergen sequences can also be "publicly available." It is not clear that a database that allows public searching, but that provides limited or no access to the underlying sequence data, is actually "publicly available."

One Panel member suggested that moving directly from consideration of database construction to questions about "triggers for significance" misses the importance of the tools used for doing the sequence comparisons. This member agreed with the agency that these comparisons have generally been carried out using the FASTA and BLAST implementations of sequence comparison algorithms, but felt that it is important to realize that both of these tools were designed to search for evolutionary relationships. In particular, the scoring systems most commonly used with these tools, such as the PAM and BLOSUM families of matrices, are explicitly derived by measuring amino acid substitution rates in families of related proteins. This Panel member pointed out that it has never been demonstrated which, if any, of the various available scoring matrices is most appropriate to the problem at hand – which is to understand how the human immune system will view the relationship between two proteins that are NOT evolutionarily related (at least not in a meaningful way). Further, this Panel member noted that it has been demonstrated that the use of matrices that assign positive values to amino acid substitutions can prevent recognition of potentially significant regions of exact match.

The Panel agreed that the use of FASTA or BLAST in the context of the recommendations of the FAO/WHO expert consultation is intended to identify situations where the potential for cross-reactivity between a query protein and a known allergen should be considered. The FAO/WHO expert consultation suggested the use of two criteria for this identification: (1) the presence of a region of more than 35% sequence identity between the query protein and a known allergen using a window of 80 amino acids and a suitable gap penalty or (2) the presence of a region of exact match of at least 6 contiguous amino acids in length. The Panel acknowledged that some details of these rules are not optimal (also see Ivanciuc et al., 2008; Ladics et al., 2006; Silvanovich et al., 2006); however, the Panel strongly believed that both rules appear to have worked well in the past. The number of situations that have required further examination as a result of the use of these criteria is quite small (only two documented cases), and thus they do not appear to represent an extraordinary burden for PIP registrants.

There was a general consensus among the Panel members that it would be premature to modify the first suggested criterion or to completely abandon the second for several reasons. First, although there is active work in progress on the potential for use of alternative criteria such as property distance (PD) values (Ivanciuc et al., 2009) or E-values (Cressman and Ladics, 2008), this work is not yet sufficiently developed for application in a regulatory environment. One Panel member pointed out that there are significant questions about the applicability of the e-value statistic in this context. The most critical of these questions is that the derivation of the equations used to calculate e-values was based on the assumption that the database involved is very large and consists primarily of unrelated sequences. It is not clear that a database of fewer than 1,400

sequences, many of which are minor variants of each other, is consistent with these assumptions.

One Panel member suggested that IgE cross-reactivity of two proteins requires that the two proteins have at least two common properties, a similar 3D fold (global requirement) and at least two similar surface regions that form common IgE epitopes (local requirements). This member felt that the use of the two FAO/WHO criteria is a reasonable approach to addressing both of these properties (Ivanciuc et al., 2008), and supported this assertion with a statistical analysis of allergens of the SDAP database (Schein et al., 2007).

One Panel member presented a detailed sensitivity and specificity analysis of the FAO/WHO recommendations using entries in the SDAP database as positive controls. For this analysis, the SwissProt database was filtered to generate a set of presumably non-allergenic proteins (negative control). This filtering first removed all entries in the SDAP database from the SwissProt entries. Next all records that (1) contained allergen related keywords, such as allergen, allergy, lipid transfer protein, profilin, lipocalin, pectate lyase, tropomyosin, melittin, thaumatin, and seed storage protein, (2) have a sequence shorter than 80 residues, or (3) belong to InterPro, Pfam, or Prosite allergen-related classes were removed. In this analysis, a threshold of 35% for the sequence identity was set to provide a basis for separating allergens from non-allergens. The number of matches found in this analysis, about 6% of the total sequences at a 35% threshold value, was not considered unreasonably high by this Panel member. This Panel member also stated that a value of 35% identity is used in the protein structure prediction community as a guide for asserting that two proteins have the same 3D fold.

The same Panel member also presented an evaluation of the second FAO/WHO criterion. A study that systematically varied window lengths between 6 and 20 identical residues was performed. When a window-length of six identical amino acids was used, 71% of SwissProt proteins were classified as positive. However the level dropped to a much smaller value of a few percent with a window length of 8. This Panel member thus felt that the two FAO/WHO criteria represent a good combination of a global and a local criterion for allergenicity. This member also stated that the importance of the second criterion has been demonstrated by work that generated chimeric proteins from two allergenic proteins involved in the pollen-food syndrome (PFS) (Klinglmayr et al., in press). This syndrome is found in a subpopulation of patients who are sensitive to the pollen allergen Bet v1 and who are also sensitive to fruits, nuts, or vegetables. However, not every Bet v 1-allergic patient shows clinical reactivity towards apple. There is published evidence that the observed clinical cross-reactivity in PFS patients is a consequence of IgE cross-reactivity between the pollen allergen Bet v 1 and the major apple allergen Mal d 1. Klinglmayr et al. generated chimeric proteins by grafting four Mal d 1 stretches (7-8 amino acids long) onto the Bet v 1 protein. The ability of this chimeric protein (BMC) to bind IgE from sera from Bet v 1 allergic patients with and without PFS was tested. Compared to birch pollen-allergic individuals, patients suffering from PFS showed significantly higher IgE reactivity with BMC (a chimeric protein), thus confirming that the local stretches could transfer clinical cross-reactivity from Mal d 1 to

BMC. This Panel member concluded that the second FAO/WHO rule would indicate the potential for this cross reactivity based on the local similarity of BMC to Mal d 1.

Another Panel member suggested that it is not entirely clear whether structure should be considered solely at the level of the intact protein and raised the point that most, if not all, food proteins can be expected to undergo some degree of degradation before consumption and during digestion. This suggests that the structure of importance might be that of peptide fragments that are less than 80 amino acids long. Further, this Panel member pointed out that researchers routinely conduct epitope mapping experiments using artificial peptides of 10 amino acids or less that retain immunologically significant structure. This suggests that, while it seems reasonable to treat a positive result obtained using the first FAO/WHO criterion as a candidate for further analysis, a negative result cannot be used to exclude the need for further sequence analysis.

One Panel member also pointed out that an alternative form of structural analysis that has been discussed involves classification of protein domains using the Pfam database. This Panel member stated that the value of Pfam information is not yet clear, particularly because it appears to be a relatively imprecise way to look at structural relationships that can be studied more accurately and in greater detail through direct comparisons. Further, according to this Panel member, insofar as knowledge of protein domain structure is of value in this context, the potential of other resources such as the Blocks, ProDom and Prosite databases should be explored. This Panel member believed that it is premature to suggest that there is a specific need for Pfam data.

Based on the fact that short artificial peptides can bind IgE from food allergic patients, and that various mapping experiments have shown that some substitutions in these very short sequences do not abolish this binding, it would seem reasonable to retain the short sequence assay as an indicator of matches that are appropriate for further consideration. To date, we are not aware of any attempt to use knowledge of actual epitope sequences or of the effects of amino acid substitutions in or near epitopes to analyze various alternative approaches to conducting the "short sequence assay."

Panel members made several other observations. First, it was pointed out that one of the public submissions indicated that an analysis of the Allergenonline.org database in early 2009 found 1.2×10^6 unique 6 amino acid sequences, 1.3×10^6 unique 7 amino acid sequences, and 1.3×10^6 unique 8 amino acid sequences in the data set. The Panel noted that these numbers are remarkable both for the fact that the number of unique sequences does not change significantly with length and that they are such a small fraction of the total universe of possible sequences. This result suggested to the Panel that allergen proteins might, in fact, have distinguishing properties that can be used in a public health risk assessment.

Second, one Panel member presented an analysis of data published by Silvonavich et al. (2006) showing what is called the probability of a match between peptides of defined sizes taken from the Allergenonline.org database and peptides of the same size in what is essentially the GenBank database. Figure 1 below graphs these

results and shows that the reported probabilities at the 6, 7, and 8-mer level are very similar, especially as compared to probabilities at the 4 and 5-mer levels.

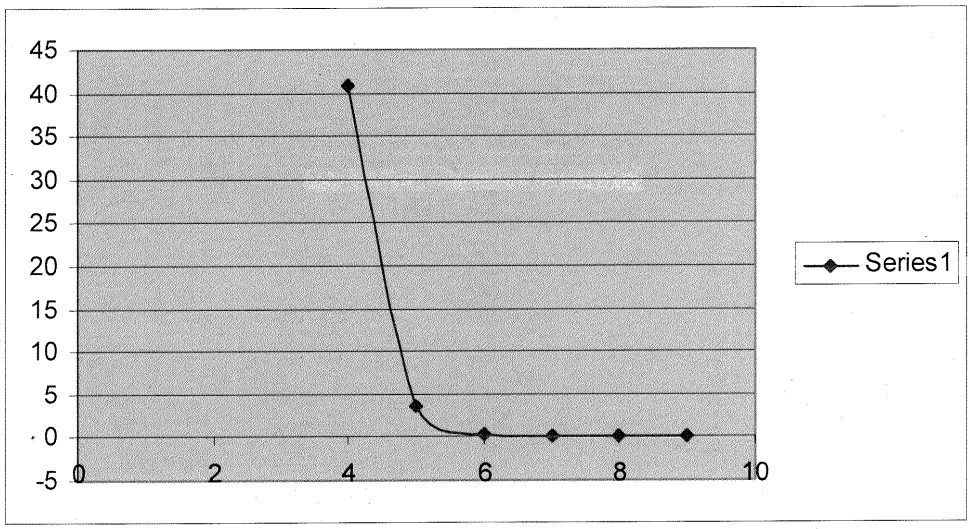

Figure 1: Probability that a peptide of defined length from the Allergenonline.org database will match a peptide of the same length in the GenBank database. The X-axis is peptide length and the Y-axis is the % of peptides of a particular size from the Allergenonline.org that finds a match in GenBank.

Third, the Panel pointed out that at least one published example exists showing how to use the "short sequence assay." In this example, reported in Ladics et al. (2006), the significance of a particular short sequence match was tested using serum binding assays. Information presented to the Panel from EPA and public comments indicate that a second example (and possibly a third) also exists. The Panel thought that these examples can be taken as an indication that the process is working as intended. In each case, bioinformatics identified a candidate for further analysis, and a particular form of further analysis was used to assess the health risk.

Finally, the Panel addressed the question of bioinformatics analysis related to protein toxins. They stated that, as complex as the situation is for allergen sequence databases, in contrast there is little that can be said about databases for analysis of protein toxins, antinutrients and "other hazardous" proteins. They acknowledged that it should be possible to develop appropriate databases using one or more sets of explicit criteria to identify appropriate sequences for inclusion. However, they were not aware of information indicating that this has been done. They thought that it should be straightforward to recognize situations that need further analysis by using the repository databases.

Charge Question C.1 – EPA has developed a draft test guideline [See OPPTS Guideline 890.3800- Synergistic Activity Test] for determining the potential for synergism for combination PIP products, specifically for registration applications that intend on citing existing toxicological data from previously registered PIP event lines. Please comment on the methodology to support the analysis for synergistic effects for combination PIP products and any additional comments on this approach- including identifying instances where the Agency would be justified to require additional testing with a combination test substance containing a mixture of two or more active ingredients for data development on human health and non-target organism effects.

Panel Response – The response to this question is presented in the form of an overview, after which a more detailed response to the question is divided into principles and comments on the specific protocol used to test for synergism.

Overview

The potential problem this question is meant to address is whether existing toxicological data for two independent PIPs that have been registered previously can be considered for registration of new products that contain both of the PIPs, with specific emphasis on how to determine whether the two PIPs are likely to interact synergistically. Moreover, would the Agency be justified in requiring additional data on human health and non-target organism effects in cases where multiple PIPs were combined in a new product. In general, studies cited below show that synergism is not easily predicted, and therefore testing for synergistic effects when two or more PIPs are combined is warranted. In cases where the mode of action of two PIPs is known and they are similar, for example, most Cry1A-F proteins of *Bacillus thuringiensis* (*Bt*), the testing for synergism similar to that outlined in the protocol would be appropriate for lepidopteran and coleopteran insects. However, where multiple PIPs that have different modes of action are being combined, the protocols would have to be tailored to test the combinations taking into account their different modes of action. For example, combinations of PIP products such as Cry1 proteins and more novel potential active ingredients, such as miRNAs (micro-RNAs) targeted against sucking insects such as aphids and leafhoppers, the PIP combination should be tested against both lepidopteran and homopteran insect targets, as well as against a selected number of non-target species. With respect to Cry1 and Cry3 proteins used in Bt crops, given their proven safety record, unless a greater than 10-fold degree of synergism is observed, there would seem to be no need to test for human health or non-target effects. However, in cases where PIPs with different, and especially novel modes of action are being combined, the Agency would be justified in requiring additional testing for human health and non-target organism effects where levels of synergism as low as five-fold are detected.

Principles

The draft Synergistic Activity Test guideline, section (a) Scope, part (1) Applicability, provides general guidance to meet FIFRA requirements. The specific guidelines are not binding on EPA or any outside parties. Registrants and applicants may

propose alternatives to the recommendations described in these guidelines, and the Agency will assess these for appropriateness on a case-by-case basis. This type of flexibility is appropriate given that in the future it is clear that many new types of agents will be developed for use in plants to control a diversity of important pests including sucking insect pests, such as aphids and whiteflies, as well as nematodes, mites, and various types of plant pathogens.

Regarding specifics, from the EPA SAP position paper of February, 2009 (pg. 8, paragraph 5), "The Agency has to date applied the following rationale to its environmental risk assessment of combination PIP products: in general, it would not be necessary to require testing of two or more active ingredients in combination, as long as the three conditions enumerated above are satisfied. Briefly, these are that (1) structure of the inserted material is conserved, (2) levels of expression are comparable to that of the parental lines, indicating that there would be no significant increase in exposure from the combination of PIP products to humans or non-target organisms, and (3) demonstration of the lack of synergistic effects. This is because the effect of the mixture of transgenic PIP active ingredients would not be greater than the addition of the effects of the PIPs tested separately. Therefore, as long as synergistic effects are not demonstrated against a susceptible target pest, no unexpected adverse effects on non-target organisms and the environment are anticipated as a result of combining two or more active ingredients."

If synergistic effects are demonstrated in future applications for combinations of PIP pesticidal substances, then the Agency would be justified in requiring additional toxicity data to establish the combined effect of the PIP substances on representative non-target species for the environmental risk assessment. Similarly, for determining effects on human health, if enhanced toxicity (that is greater than additive) is demonstrated, then additional Tier I Acute Oral toxicity tests at the limit dose would be required for the combination of active ingredients. The latter comment is based on a recommendation by the 2004 SAP using a Cry1Ab plus Cry1F combination as an example. In this case, as far as is known, the basic mode of action of these two proteins is the same. Yet even in cases where the basic mode of action is the same, significant levels of synergism have been observed. For example, against larvae of the gypsy moth, *Lymantria dispar*, Cry1Aa plus Cry1Ac at a 1:1 ratio exhibited a level of synergism of 3.8-fold compared to Cry1Aa alone; at a 1:2 ratio, 7.3-fold; and at 1:4 ratio, 4.9-fold. Interestingly, the same combinations showed no evidence of synergism against larvae of the silkworm, *Bombyx mori* (Lee et al., 1996). This illustrates the difficulty of testing for synergistic effects and deciding what range of non-target species should be tested. In general, if non-target organisms will be exposed to PIP combinations with levels of synergism less than ten-fold against the target organism, there would appear to be no need for non-target testing.

Importantly, it is further noted in the EPA position paper on this guideline that the Agency reserves the right to require toxicity tests on the combinations as appropriate, for example, where a novel source of a transgene is used in combination with known toxins, or where toxins which have different modes of action are combined (page 9). This is an important point because where high levels of synergism have been noted, it has typically been with toxins that have different modes of action. For example, in the case of Cry4

and Cry11, mosquitocidal proteins of *B. thuringiensis*, mixtures with the *Bt* Cyt1A protein are 10-fold or more toxic than the individual toxins, depending on which mosquito species they are tested against. In this case, this is likely due to the different modes of action of the toxins; Cry proteins require glycoprotein receptors on midgut microvilli, whereas Cyt1A does not – instead, it binds directly to the lipid portion of the microvillar membrane, assisting the binding and penetration into the membrane of Cry proteins. Thus, it can be anticipated that some combinations of PIPs may have similar synergistic effects if they have different modes of action. In addition, even if the mode of action is the same, for example in the case of different *Bt* Cry proteins, additional non-target tests may be warranted, especially if the degree of synergism is high, for example, greater than ten-fold of what would be expected from additive effects. In addition, not all Cry proteins have the same mode of action.

With respect to the types of additional studies that would be required where a substantial level of synergism is detected, studies of the effects on a certain range of non-target invertebrates, namely beneficial insects, would be warranted. Moreover, whereas there may be no need for tests on human health effects owing to the substantial safety record now available for several Cry PIP proteins, the relatively small costs of doing a limited amount of Acute Oral testing against mammalian species would also be warranted, especially if the degree of synergism were high, e.g., ten-fold or greater than that which would be expected from additive effects. This would provide additional assurance of safety to humans, to the extent that such assurance is possible from such tests.

Synergistic Test Protocol.

In general, the Panel found that the specific protocol presented in the draft guideline is overly prescriptive. It very much reads like a protocol for testing synergism of protein toxins active against lepidopteran insects, and possibly some coleopterans. It would not work for pests like aphids and nematodes. Thus, one way to modify the guideline would be to identify the protocol as an example of one appropriate for lepidopteran pests that feed on leaves or seeds. It could then be stated that other protocols would be considered by the Agency for testing synergism, especially for combinations of novel PIPs, noting that regardless of the protocol, the specific protocol presented provides examples of the level of detail that would be required, as well as the types of statistical tests that would be required to validate the results. Overall, as noted in the introductory statements to the proposed test guideline, the Agency and pesticide registrant should come to agreement before testing on specific protocols for the test pest, be it an insect, non-insect invertebrate, or a plant pathogen.

With respect to the PIPs, while surrogate hosts (e.g., bacteria) could be used as the source of test material, fresh or lyophilized parental plant tissues could be used if they are available and suitable for bioassays. An advantage to using these is that other plant proteins and secondary compounds that may be involved in synergism would be present in the plant materials. This way, direct comparisons could be made between the parental plant tissues and those that contain a combination of two or more PIPs.

In regard to the dilution series to be tested, this seems overly complicated, especially where 3 or more combinations are to be tested. If each dilution is tested and sufficiently replicated, the combination of treatments that would be required would be substantial. After a range-finding test, it might be better to first test combinations of 1:1 at LC50 levels and then decide which additional combinations and dilutions are required. At least three replicates should be used for each test concentration, and in each replicate, at least 10 larvae or nymphs should be used. If control mortality is high, then additional replicates should be conducted, or the number of larvae in each replicate increased.

The Panel also noted that tests/replicates should be carried out on different days to control for day-to-day variations in test species physiology and that the meaning of the statement "test species should represent a continuum of high to low susceptibility" should be clarified.

The Panel suggested including the following additional possible references on synergism and additive effects to the reference list in the proposed Guideline:

> Wirth, M. C., G. P. Georghiou, and B. A. Federici. 1997. CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. *Proc. Natl. Acad. Sci.* U. S. A. 94:10536-10540.
>
> Crickmore, N., E. J. Bone, J. A. Williams, and D. J. Ellar. 1995. Contributions of individual components of the *d*-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. *FEMS Microbiol. Letts.*131:249-254.

Charge Question C.2 – EPA has developed soil microbial community toxicity tests for plants that are modified to inhibit microbial growth (e.g., a gene is inserted into a plant that exhibits a microbiocidal mode of action against a plant pathogen) [See OPPTS Guideline 890.3850 Soil Microbial Community Toxicity Test]. Considering the natural fluctuation of microbial populations in the soil ecosystem and the inherent variability in extracting representative soil samples, please comment on limiting testing to examining effects on activity of beneficial soil microbes, specifically carbon and nitrogen cycling at the soil microbial community level (soil is analyzed for NH3 and NO3 content to establish ammonification and nitrification values, respectively, and for CO2 efflux, in the presence and absence of the stressor). Please provide a basis for your answer.

Panel Response – The Panel agreed on the importance of developing soil microbial community toxicity testing for plants that have PIPs targeted at inhibiting microbial growth. They expressed appreciation for the complexities involved in drafting guidelines for developing microbial assays that will yield useful information in assessment of PIPs in soil, due to the myriad of microbial groups, activities, and functions and the inherent variability found in soil. In addition, they realize the difficulty in drafting toxicity tests

for plants that are still under development, and attempting to predict toxicity of future genes or traits.

The EPA position paper states that it intends to confine its testing to measurements of microbial respiration and beneficial nitrogen and carbon cycling processes. Specifically, the OPPTS Guideline 890.3850 requires definitive testing when the test substance results in greater than a 50% reduction of ammonification, nitrification, and CO_2 evolution. The Panel noted that it is important not only to consider toxicity of a PIP compound to the microbial communities (causing a reduction in these measurements) but also the stimulation of certain portions of the community that may then cause a significant increase in these functions. Examples of negative consequences of a stimulation in the microbial community are easily found. For example, if the PIP compound is an appropriate carbon source for a plant pathogen, then this pathogen will have a competitive advantage and could predominate in that system, altering ecosystem function and plant health. The Panel concurred that soil microorganisms play critical roles in carbon and nitrogen cycling. However, they noted that these are only two of the important roles they play in soil ecosystem functioning, and limiting testing to examination of the suggested broad functional measures may be inadequate. The Panel based this response on several factors:

First, the EPA position paper clearly outlines the critical roles soil microbial communities play in soil ecosystem functions. Several key functions are mentioned in OPPTS Guideline 890.3850, such as organic matter turnover; carbon, nitrogen, phosphorus and sulfur cycling; and regulation of plant pathogens. The Panel noted that there are other key roles of the microbial communities that should be mentioned, including the following:

i. Plant growth promotion, either through direct release of growth regulators, biocontrol of pathogens, nitrogen fixation, increased access to phosphorus and water.

ii. Soil aggregation, and soil physical structure. This is a key factor when examining soil carbon sequestration, and plant health due to the impact on water holding capacity and soil aeration.

iii. Degradation of xenobiotic compounds. Any protein released into the soil system will be broken down by microorganisms to fulfill nutrient and energy requirements.

Second, the EPA position paper justifies its consideration of functional activities rather than variation in specific genera due to the "uncertainties in determining cause and effect in variations in soil microflora". It is true that the link between taxonomy of soil microbial communities and function is not entirely clear. Increased diversity does not always equate to increased functioning. Microorganisms are known to show phenotypic plasticity, so often multiple groups of organisms are present that can complete the same soil function. Therefore, altering the composition of the microbial community will not

necessarily alter soil ecosystem function. However, it is still important to understand changes in community composition, because, as in higher level organisms there are keystone species that have control over entire soil food webs. For example, Klironomos and Kendrick (1995) showed that arthropods typically feed on saprophytic fungi, but if those fungi are not available, they will feed on mycorrhizae, and if this community is depleted it will have an impact on the health and growth of the associated plants. In this case, testing only C and N transformations would not catch any changes in the system because the total CO_2 would likely be unchanged. However, in this example, soil community differences would occur because of elimination of a predator-prey interaction and these changes would impact the soil ecosystem function, including higher trophic levels, e.g., plant health. Several studies on interactions of introduced substances with soil microbial communities in cropping systems reveal that the most pronounced effects are detected with specific genera or species of microorganisms rather than with broader measurements of soil microbial respiration, biomass, etc. In a review on impacts of disturbance on soil microbial communities, some of the older literature (e.g., Wardle 1995), suggests that measurement of response by functional groupings may be problematic because individual taxonomic species are likely to be more sensitive to disturbance rather than an entire functional group comprised of multiple taxonomic groups. If PIP introduction is considered an external ecosystem disturbance, it is expected that this will, more than likely, have little or no effect on soil microbial biomass and broad functional activities such as soil respiration and perhaps soil enzymatic activity.

Third, the Panel noted that since the PIP products in question are engineered to produce fungicidal or bactericidal substances, depending on concentrations upon entry into soil, one might expect to observe detrimental effects on soil microbial groups and/or soil functions. The microbial groups these new PIPs affect should dictate the appropriate test variables, indicator groups or species. If the PIP is fungicidal, tests for impacts on the population of symbiotic arbuscular mycorrhizal fungi will be needed. Similarly if a PIP is bactericidal, the concern would be the targeted microbial (bacterial) groups. It is short-sighted to focus on differences in CO_2 or NH_3 in these cases.

The Panel also provided specific comments about the choice of the toxicity tests outlined in the OPPTS Guideline 890.3850. The assays under consideration are in general use in many soil biology studies; however, it is important to note their limitations, especially in the context of assaying impacts of substances introduced in the environment.

Respiration assays are general at best, they assess very broad functional activity, and may mask any sensitive group involved in decomposition. This may be the consequence of using "bulk soil" in mesocosms rather than root-influenced (rhizosphere) soil that has higher diversity of microbial taxonomic groups. This may also contribute to findings of some reports on GM crop assessment that indicate no effect using respiration assays (i. e., Liphadzi et al., 2005). It is important to be aware of the potential for overlooking possible subgroups involved in C transformation that might be impacted by a PIP, but the effect is masked within the total CO_2 efflux data collected for the general microbial community.

The Panel supported the selection of *ammonification* as a critical test, as this somewhat focused on a selective group or segment of the soil microbial community, although other sub-groups involved in specific N mineralization from proteins (proteases) or amino acids (deaminases) might be more sensitive to the PIP.

Nitrification is a good assay to include in monitoring the total N cycle, but basing this on the end product (NO_3) is problematic. There is not a lot of information on other potential sources of NO_3 or on actual nitrification activity. Soils vary widely in nitrifier populations and we know that activity is likely mediated by more than the one species for each nitrification step. *Nitrosomonas* and *Nitrobacter* are generally recognized as the major genera; however, there is evidence of contribution to soil nitrification by other species, such as *Nitrosospira.* More importantly, agroecosystem activities and previous management, and/or soil NO_3 level as well as soil pH, will influence nitrification activity. For example, many cultivated soils that have received steady amounts of inorganic N fertilizers tend to have higher nitrification activities (perhaps due to residual NO_3 levels) than grasslands, forest, and natural ecosystems. Thus, such high activity in a test soil of cultivated agricultural origin may mask any effect of an introduced PIP. Based on previous work, it may be problematic to consider NO_3 analysis comparable in terms of reliability or confidence to other methods for soil nitrifying activity, i.e., nitrifier MPN (most probable number), specific probes based on genes coding for ammonium oxidase and nitrite oxidase. It may be useful to consider detection of microbes capable of nitrification instead of measures of nitrate levels.

Recently Kowalchuk et al. (2003) recognized the limitations of general measurements such as soil microbial biomass and respiration for describing potential genetically-modified (GM) crop-induced effects on the microbial community. They proposed that a polyphasic microbial analysis, comprised of selected indicator groups and activities combined with general assays including microbial diversity analyses (molecular or physiological), would yield a more comprehensive and informative assessment of potential impacts of GM crops. The Panel suggested that this approach may be applicable to evaluate soil activity of introduced PIPs. A multiphasic approach may provide a more comprehensive analysis of microbial community structure and function in response to PIP exposure, especially if initial microbial assays reveal complex and varied responses.

The EPA position paper comments directly on approaches to determine soil microbial diversity, i.e., molecular biological techniques, and states they show promise in helping understand impacts of GM crops on soil microbial ecology. The position paper dismisses the method as an appropriate assessment technique due to the current lack of a definition of a taxonomic unit. The Panel suggested that dismissing this commonly used technique is premature. Soil or rhizosphere bacterial diversity evaluations based on physiological or molecular analyses often differs when comparing conventional to GM cropping systems, and these differences may parallel variations in composition and activities of "key indicator" groups or functions. There is some indication that different groups within the microbial community vary in sensitivity to introduced transgenic proteins (i.e., cp4 EPSPS) (Hart et al., 2009). It is widely reported that decreased microbial diversity in many ecosystems (either natural or agricultural) is a concern

because high diversity is essential in maintaining a stable ecosystem and plant productivity (Grayston et al., 1998). In addition, it is important to note that there are many well developed techniques targeting functional genes such as those that target nitrification (*amoA*) or denitrification (*nirs, nirK, nosZ),* that can be used to indicate soil function, and the diversity of the group of organisms responsible for that function, therefore molecular analyses are no longer limited to taxonomic studies, and the stated concern of the EPA regarding the definition of a taxonomic unit is no longer valid.

Finally, it is important to note that studies examining the impact of a GM plant on soil microbial biodiversity often show differences between a GM and non-GM isoline. However, these differences are often dependent on field site and are either due to soil type or environmental variables. Further, changes are often temporary and dependent on the presence of the plant. Therefore, a simple measurement of a change in biodiversity is not necessarily indicative of an environmental concern. Similarly, when measuring alterations in soil function it is important to keep realistic perspective in mind. The significance of any metabolic changes noted may need to be put into perspective by comparing them to changes caused by accepted agronomic practices, such as crop rotations—particularly, seasonal and yearly changes in rotating conventional leguminous crops with non-leguminous crops (e.g., soybeans and corn)—and pesticide and herbicide use. One Panel member noted that lessons we have learned from the first PIP tested, such as the Bt systems should be considered. In Bt systems, hazards can be demonstrated in laboratory studies using non-target insects and other invertebrates. However, these risks were deemed acceptable after subsequent studies carried out on Bt crops under normal agronomic growing conditions showed that the risks were minimal, and that the benefits, such as reduced use of chemical insecticides and increased worker safety, far outweighed any potential risks. In this light, it is important to put the results of toxicity tests on soil microbial communities in perspective, possibly through comparing to the toxicity of the standard, non-PIP agronomic practice.

The Panel made the following specific comments regarding testing beneficial microbial processes related to C and N cycling, and the Toxicity Tests described in the EPA Guideline:

One Panel member suggested changing the Soil Microbial Community Toxicity Test OPPTS 890.3850 to a tiered approach , as generally followed by the EPA. The definition of harm in this test should include not only a decrease, but also a significant increase in activity, and endpoints of tests should be clearly defined. In addition, tests should be related to laboratory studies of soil persistence or field studies of accumulation which have been required for PIP registration in the past.

Attempting to duplicate soil conditions and microbial communities in the laboratory is extremely difficult because microbial communities, even agricultural ones, are very dynamic. Thus, any results obtained from laboratory studies could be challenged on the basis of whether they represent solid science, and could be easily criticized by any party that disagreed with interpretations of the results. In light of this, it might be best to

attempt to develop techniques that could be used in field conditions by comparing a specific multiple-PIP crop directly with its non-engineered isoline.

Thorough characterization of the test soil(s) is necessary, including ecosystem of origin, texture, "rhizosphere" vs "bulk" origin, and storage conditions (time of collection, conditions when collected [air-dry, field moist], temperature, time since field collection). It is important to remember that each soil's physico-chemical properties already influence microbial activity, and when PIPs are introduced into soils those properties will also affect microbial response to the PIP.

The Panel also made the following specific comments and recommendations related to the soil testing protocol:

1) **Total N Analysis**. An analysis of total N should be required and relative levels of ammonification and nitrification compared with total N in system. This test could be an element of the analysis of soil organic matter quality. The N analysis should be conducted on soil before and after alfalfa meal amendment; origin (standard from supply company or lab prepared material) and N analysis of alfalfa meal should also be determined. In addition, a no-alfalfa-control is needed in order to see the natural variation in mineralization rates in the soil. These soil functions are microbial processes, and therefore have a large inherent variability.

2) **Total Organic Carbon.** A more quantitative and standardized analysis of total organic C, rather than or in addition to soil organic matter expression (no finite chemical composition available for soil organic matter) is recommended.

In this test, the choice of soil will be critical. Different soils will have very different responses. The range of soil options for the test is huge and they will definitely impact the success of the test, especially considering that most mineral soils fall within a 1-8% organic matter range and this is without the consideration of the impact of clay content.

3) **Metabolism and moisture stabilization.** Generally one would sieve the soil to less than 2 mm and then pre-incubate at 25°C to 40-50% moisture-holding capacity for 7 days in order to permit metabolism and moisture to stabilize before the start of the experiment.

The suggested protocol gives a lot of choice with respect to incubation range. These choices will have major impacts on the results of the test and should be controlled. Incubation temperature will be critical in this test. Therefore, it may not be appropriate to give a range of potential temperatures. For instance, if the case is made that the soil is going to be cool, such as 10°C, then microbial metabolism would slow down, and the experiment would need to be extended.

4) **Plant residues.** Presentation of the chemical protein itself will be rare. Likely, the microorganisms would contact plant residues containing the protein. Therefore, it may be more realistic to present residues with or without the PIP.

5) **Soil function trigger.** Under the proposed guidelines, the 'definitive test' for soil microbial community toxicity is only triggered if reduction of ammonia, nitrification, and CO_2 is less than 50%; the Panel suggested it is important to understand an increase in these soil functions also.

Charge Question D.1 – Gene flow has been distributing various naturally occurring genes between sexually compatible species for millions of years and some of these genes encode traits for plant disease and insect resistance mechanisms. Various methods have been used to study the impacts of natural gene flow. Assuming a case wherein hybridization and introgression of a transgene expressing a pesticidal substance occurs between crop and wild species:

a.) The EPA asks the Panel to discuss whether it is possible to evaluate, in part, impacts of a gene flow event by gathering data on target (pest) species which are associated with the wild species (transgene recipient).

b.) The EPA asks the Panel to discuss whether the gathered data will allow estimating the degree to which resistance to these target species may influence the population dynamics or invasiveness of the wild relative.

c.) The EPA asks the Panel to discuss whether empirical data regarding the target species (e.g., fungi, insects, etc) and non-target species (e.g., pollinators, detritivores) associated with the sexually compatible wild relative have the potential to inform about risks to the SCWR population and the associated community.

d.) The EPA asks the Panel to discuss whether an understanding of the potential effect(s) of introgressed transgenes on basic plant habit, phenology and physiology provide a basis for assessing potential impacts following a gene flow event.

Panel Response – D.1a: Discuss whether it is possible to evaluate, in part, impacts of a gene flow event by gathering data on target (pest) species which are associated with the wild species (transgene recipient).

The Panel assumed this question was being asked because the target species is also some form of pest on the sexually compatible wild relative (SCWR) and that one might, therefore, want to determine whether the SCWR would be affected positively or negatively by acquisition of the PIP via gene flow. It is certainly important to know the association between the SCWR and the target species, but it is not sufficient to rule out or predict adverse effects.

The association between the target species and the SCWR can vary spatially and temporally; therefore, in order to begin to assess the likelihood that a given PIP could

cause harm in the SCWR, it is important to collect data on the association between the target species and the SCWR populations in many populations over more than one year. Data to examine such variation can include the following:

- Documentation of the presence of the target pest species in SCWR populations and of the geographic range of the SCWR-target pest association.

- Assessment of the abundance of the target pest in the wild over space (i.e., in various populations of the SCWR) and over time in some of these populations. This would help estimate the potential importance of the pest/disease in regulating the wild plant populations. In addition, assessment of the frequency and intensity of infestations in natural conditions will aid quantification of the selection pressure on the PIP.

The difficulty for cases where the presence and/or abundance of the target species varies over space and time is to determine how much data to gather before one is confident that one understands the potential impact of the target pest or disease in wild populations. For example, a disease may be rare most years but create an epidemic when the environmental conditions are favorable, which may occur on time scales approaching a decade or more. If the disease has a strong impact on the SCWR's fitness, then these relatively rare epidemics can be important. Therefore, such data must always be interpreted with caution as pest outbreaks and disease epidemics in wild populations do not occur as frequently as in agricultural systems.

Unfortunately, information about many target species and their association with the SCWR is not likely to be in the literature since entomology and plant pathology have historically focused on interactions with crop species. In some places there are significant records from naturalists on the distribution of SCWR and associated species that could be of use.

Data on the target species associated with the wild species is necessary, but not sufficient, to determine the impact of a gene flow event and the effects of a given PIP in the SCWR populations under natural conditions. One would also want to know the degree to which the target pest regulates and poses a selection pressure on the SCWR's populations. This depends on the abundance of the target species in wild populations and on its effects on the fitness of the SCWR individuals in the wild. High abundance of the target species and high impact on fitness of the SCWR will increase selection for an introgressed PIP in the SCWR population, resulting in increased frequency of the PIP gene(s). It is this change in the frequency of the PIP in the SCWR population that could ultimately have greater consequences. For example, the PIP could increase the population growth rate of the SCWR populations and increase the SCWR's ability to compete with other plant species in the community. Such modifications of the dynamics of the system could increase the potential for invasiveness of the SCWR.

To assess the potential effect of the PIP on the wild species, we suggest that naturally occurring field populations be monitored for presence of the target species and its impact on the wild species. This would not be without its challenges. Levels of infestation in natural populations vary from year to year and among locations, and resistance to the target species often will also vary in time and space. In addition, interactions with other parts of the community can cause the effect of the target species to vary (e.g., disease can be especially bad when insect damage rises). For instance, if an SCWR has little or no disease from the target species, could it be because the SCWR already has resistance to the target species or possibly because innoculum levels are low in a given region for other reasons? Moreover, the relationship between levels of infestation on a plant and plant fitness or an estimate of plant fitness, such as reproductive output and/or survival is necessary to determine the impact of the target species on the wild species (and therefore the potential impact of the PIP in the population). It could be difficult to determine how much data to collect before one is confident that one understands the potential impact of the target species on SCWRs. Nevertheless, as long as it is possible to see damage done by the pest, these data should be collected. This discussion is expanded to involve experimental plantings in the Panel's response to Charge 1b.

Panel Response – D.1b: Discuss whether the gathered data will allow estimating the degree to which resistance to these target species may influence the population dynamics or invasiveness of the wild relative.

This is a difficult question, which gets at one of the major challenges for evolutionary ecology and ecology in general. Predicting invasiveness is clearly one of the salient questions of our time, particularly because new invasives continue to alter our natural ecosystems. We are much better at determining *a posteriori* why a species probably became invasive, than predicting which species or populations will become invasive. Although a precise set of characteristics of invasives is elusive, the conditions resulting in a new invasion might include some combination of increased population growth, increased competitive ability, and increased spread/dispersal ability. Mechanisms for the development of invasiveness include the enemy-release hypothesis which specifically addresses the possibility that release from disease or insect pests in the introduced range could increase the chance of a plant becoming invasive. We also know from experience that becoming invasive often requires a "lag time" of greater than 50 years, so short term experiments under one set of conditions or with one genetic background might not yield definitive information.

Before the PIP can influence the population dynamics or invasiveness of the wild relative, it must increase in frequency in the SCWR populations. Such an increase in frequency could result if the PIP provides a selective advantage to SCWR individuals under natural conditions. Alternatively, an increase in PIP frequency could result from genetic drift or recurrent gene flow (i.e., genetic swamping) from the crop fields into the SCWR populations which could maintain the PIP at significant frequencies in the SCWR populations. Levels of expected gene flow could be inferred by examination of the mode of pollination, the breeding systems of the SCWR and the crop, the likely distance of

SCWR populations to crop fields, and the likely crop rotations employed. Genetic drift would play a greater role in SCWR with a metapopulation structure and small population sizes. Under such circumstances, genetic drift could fix the PIP allele in some populations while driving it to extinction in others. Thus, determination of the population structure of SCWR in the wild would indicate whether genetic drift is likely to affect the frequency of a PIP in those populations.

In the Panel's response to Charge D.1a, the relationship between SCWR fitness in natural populations to target pest levels was discussed. Here, the Panel investigates the same issue from another perspective: what is the fitness of SCWR plants, with the PIP introgressed, relative to those without the PIP in the presence or absence of the target species? The answer to this question would contribute to an estimation of the selection for or against the PIP. The difference between PIP-containing and PIP-lacking plants in the presence of the target species determines the selective advantage provided by the PIP to the SCWR. The difference between the two in the absence of the target species determines whether there is a cost to carrying the resistance. These data permit calculation of the relative fitness of the transgenic SCWR in natural populations and help clarify how the frequency of the transgene may change in the population over time. Fitness measures ideally include lifetime survival and seed production. Such measures are easier to obtain for annual plants, but can be quite difficult to measure in long-lived perennials. For the latter, fitness of the plant is often estimated over one or two years. Indirect measures are also used, including estimates of flower production, rather than seed set, or for particularly long-lived species using plant size as a surrogate for fitness. The less direct the measure of fitness, the less accurate the estimate of relative fitness of transgenic and non-transgenic wild plants.

Fitness studies focusing on the effects of the PIP with and without target pest pressure can sometimes be difficult in practice. First, there is a risk associated with using PIP-containing SCWR plants for experiments in the wild since this experimental approach could lead to unintentional escape of potentially invasive plant material. When feasible, measures could be taken to eliminate gene flow out of experimental plots, but zero gene flow is difficult to achieve. To avoid having to use transgenic SCWRs, some investigators have mimicked the activity of the PIP by controlling for the target pest manually or using pesticides despite these techniques' inability to assess all genetic effects of the PIP, including costs and pleiotropy. Second, under natural conditions, many factors can affect the fitness of the plants besides the PIP. These conditions are sometimes controlled for by performing experiments in mesocosms, greenhouses or growth chambers; however, experimental demonstration that the PIP does or does not provide a strong selective advantage to the SCWR under greenhouse or growth chamber conditions does not necessarily indicate an identical advantage or disadvantage under natural conditions. For example, disease or target pest levels may be lower in wild populations than they were in the greenhouse, so it would be less advantageous to a SCWR plant to carry the PIP in the wild than the greenhouse study would have indicated. Alternatively, there could be a non-target pest commonly found in natural SCWR populations that strongly reduces SCWR fitness despite the small advantage the PIP provides with respect to the target species.

An increase in the frequency of the PIP in the wild population does not necessarily imply a change in the population dynamics and invasiveness of the wild species (Here, we interpret population dynamics to denote studies of the growth rate of populations (λ) over time; at the individual plant level it represents the absolute fitness of a plant.) If a target species regularly infests SCWR populations across its range and tends to significantly reduce survival or seed production under natural conditions, a PIP for resistance to the target species would likely have an impact on fitness and population dynamics. In this case, one might be fairly confident predicting harm due to gene flow. Still, even this situation is complicated because changes in survival and seed production do not necessarily correlate to changes in population growth or definitively determine the risks for invasion. It is easier to measure increases in seed than it is to predict their impact. For example, it is possible that increased seed production would simply make competition at the seedling stage more intense, without a related increase in numbers of adult plants (i.e., density dependent effects). More seed could also attract more seed predators, ameliorating the potential increase in spread. In another scenario, a transgenic trait could impact competitive ability, which in turn could improve the average ability of a plant to survive from seedling stage to reproduction possibly increasing λ, as well as indicating a risk for increasing invasiveness. It is important to note, however, that such complications only occur because we often can only obtain an incomplete or indirect measure of lifetime fitness.

To predict invasiveness or alterations in population dynamics, studies would need to evaluate the relationship between the target species and wild species in a way that elucidates factors that could affect population dynamics or invasiveness in natural and agricultural systems. Here, experimental approaches may be most helpful. These studies would compare the demographic dynamics of SCWR populations carrying the PIP (or mimicking PIP expression) to SCWR plants without protection in the presence of the target species. Studies of λ use information on seed production and on the likelihood that a plant in the population survives to various stages of the lifecycle. These demographic measures can then be used to assess the rate of population growth (which can be negative) and the life stages that most strongly impact growth. Again, if these experiments were only done in the greenhouse, they might not elucidate the expected effect of the PIP on λ under natural conditions since many factors, including community composition and genetic variation influence lambda in wild populations. Thus, to fully understand the impact of PIP on λ, studies of its effects could be required in multiple wild populations.

Apart from these demographic studies, it would be important to more directly assess the degree to which populations are seed limited (i.e., population growth or spread and dispersal do not necessarily increase with increased seed production). We also need to explore what factors could increase a plant's ability to disperse its seeds, colonize, and spread. Such research areas are still in their infancy. In sum, predicting population dynamics and invasiveness is difficult in any system. Experimental determination of how a PIP alters λ, population size, and colonization could provide some guidance for expected effects.

Two cautionary notes: Which selective agents (pests) need to be studied on the SCWR are not always immediately evident. For *Bt* sunflowers, stem boring insects that influenced fitness were not easy to see (Snow et al. 2003). Second, it should be acknowledged that field work in one year cannot predict the response of plants or pests in all years. The absence of disease in one year or location does not mean that a given population is never challenged with disease. This encourages data collection from as many populations, years, and locations as possible to develop a sense of the overall impact and variation in impact of the PIP in a SCWR.

Panel Response – D.1c: Discuss whether empirical data regarding the target species (e.g., fungi, insects, etc) and non-target species (e.g., pollinators, detritivores) associated with the SCWR have the potential to inform about risks to the SCWR population and the associated community.

Only by knowing the overall effect of the PIP in the SCWR on the target and non-target organisms (NTOs) in a community could one potentially predict the consequences of gene flow (see the response to Charge D.1a above). As mentioned before, the frequency of the PIP would have to become common in the population via selection, recurrent gene flow, or drift before it could have many indirect effects on the community. Complications arise for assessing the impact of gene flow when considering the community in addition to the SCWR. The cascading effects of crop-wild gene flow, the number of actors involved, and the interactions among those actors increase the complexity of the analysis. Considering how little is known in most communities about what determines species habitats and what the most significant evolutionary pressures are on the species, a single study of the target pest and NTOs also affected by the PIP would not likely strongly predict how gene flow would affect the community at large. Interactions could be complex since the PIP could affect beneficial species, thus indirectly affecting the SCWR and other components of the community.

A first step towards understanding community-level effects would involve identifying the members of the community (especially those likely to be susceptible to the PIP) and assessing interactions among NTOs, among NTOs and their hosts, and among hosts. This would inform our understanding of how the community might be affected by PIP-induced changes in the NTOs. For instance, if some susceptible NTOs appear to play a role in suppressing the SCWR population numbers or providing essential services (e.g., pollination), then the PIP could alter the population dynamics of the SCWR and consequently its competitive ability with other host species.

There are three main scenarios here which vary in the presence of NTOs that are susceptible to the PIP and the role the NTOs play in SCWR plant fitness. They are as follows:

1. If there are no NTOs associated with the SCWR that are susceptible to the PIP, it is unlikely that the PIP will alter SCWR population dynamics or invasiveness above and beyond the effect on the wild species of

suppressing the target species. However, this also signals a situation where the SCWR is not regulated by any species other than the target species. Thus, the PIP could have a surprisingly large effect on the fitness of SCWR individuals and increase their ability to compete with other host species.

2. If susceptible NTOs have a positive effect on the SCWR growth, development, or fitness, then the PIP could negatively impact the SCWR populations (possibly lowering λ), which could raise conservation concerns. However, the precise outcome depends on whether the positive effect of the PIP on the SCWR through control of the target pest outweighs the negative impact on the SCWR by PIP suppression of beneficial NTOs.

3. If susceptible NTOs have a negative effect on the growth, development, or fitness of the SCWR, then the PIP would be more likely to have a positive impact on fitness, population dynamics, and/or invasiveness. There would then potentially be a synergistic effect on the SCWR since the PIP could then increase SCWR fitness via suppression of both the target and non-target pests. Under such circumstances, we might expect a greater probability that the PIP increases in frequency, and thus increases its population size, its competitive ability with other host species and its potential for invasiveness.

But these scenarios are simplified as they do not look at the effects of NTOs susceptible to the PIP on other host species in the community and at the competitive abilities between these host species and the wild species. Gathering data on the presence of NTOs associated with SCWR and examining how they affect the SCWR and other host species in the community will help determine which other community members, besides the target species, are likely to influence the change in PIP frequency in the SCWR population. Such information may enhance predictions of altered SCWR population dynamics.

To go from this qualitative understanding to more quantitative insight would require detailed experimental data on the structure of and interactions within the SCWR community, especially those most likely to be affected by an introgressed PIP. Though difficult to predict, past experience manipulating ecological webs (e.g., via introduction of biocontrols or eradication campaigns) suggests that knowing something about these relationships may help avoid major problems. Additionally, some NTOs, such as pollinators, can also facilitate or hinder the movement of PIPs among populations of SCWRs.

Panel Response – D.1d: How does understanding the ways that introgressed transgenes could affect basic plant habit, phenology, and physiology allow for assessment of impacts?

Future PIPs could have important effects on a SCWR's competitiveness by altering size or stature, shape, or the environmentally dependent and temporally variable

patterns of development and growth. All of these alterations in the SCWR could affect invasive potential via effects on population growth and spread. Although more traditional PIPs, such as insect or disease resistance, could impact these kinds of characteristics through pleiotropy, epistasis, insertion effects, or as a consequence of changes in fitness, growth regulating PIPs would manipulate these characteristics more directly. (Crop hybridization with SCWRs can also affect some of these characteristics, even without PIPs.) Of course, if the PIP influences plant growth, phenology, or physiology, it is important to understand the effects on the plant and how those changes might affect the ecological community. It is not sufficient to know what effect the transgene has on basic plant habit and physiology; one must also understand how such change in phenotype translates into differences in fitness relative to SCWR individuals with an unaltered phenotype.

Despite the more general nature of the traits affected by growth regulating PIPs, the same kind of studies described for questions D.1a-c would be necessary for exploring effects on fitness, population dynamics, and competition. Here we discuss PIPs affecting habit, phenology, and physiology separately.

The effects of a PIP that influences habit would depend crucially on the same factors identified in D.1a: the expression of the PIP in the SCWR, any interactions with other alleles controlling habit already present in the SCWR, and the importance of the PIP characteristic in determining the SCWR's fitness.

As described above, if the PIP product ultimately acts to increase the fitness of the SCWR, one would expect the habit of the SCWR with an introgressed PIP to be either larger or more persistent, i.e., the SCWR would increase its seed output (if primarily sexual) or its dominance in the community through growth. Where the SCWR is already weedy, increased fitness could clearly be a problem. Where the SCWR is not weedy, increased fitness may or may not translate into a change in population dynamics. Studying the demographics (λ) of SCWR populations with and without the PIP could be informative about the effects of such habit shifts. An increase in λ suggests a potential increase in invasiveness especially when associated with an increased ability to spread to new habitats. The impact of the PIP on NTOs is also important to examine. If the PIP lowers the fitness of the SCWR, but is maintained in the SCWR population by recurrent gene flow from the crop, the SCWR's habit might be diminished. If the SCWR is an agricultural weed, this could indicate that it will be less of a problem for farmers to control. If it is a non-weedy species, this could indicate that populations could decline, causing conservation concern—but that will depend on the degree to which it was previously considered a pest and the degree to which the transgene is negatively affecting the habit.

Knowledge of changes in the SCWR's phenology due to expression of the PIP could be useful in assessing the effects of gene flow, but by itself would not likely be sufficient to allow full understanding of the impact of gene flow on the SCWR and the ecological community. Phenological changes could alter gene flow potential (crop-wild and wild-wild) or alter characteristics that are important determinants of fitness. If the

PIP modifies the phenology of the crop or tree so that it now flowers at the same time as the wild species then this will increase the chances of hybridization. On the other hand, it might decrease the overlap of flowering times and limit the chances of hybridization. Thus phenology can control the levels of gene flow into a SCWR. However, it can also have a separate effect on the SCWR once it is present. For example, within the introgressed SCWR population, if plants with the transgene grow faster, they can shade out the other plants in the population and be favored (higher reproduction). Faster growth rate could then influence other plant species growing in the area as the PIP plants newly shade out some and no longer interact with others (i.e., if growth phase occurred earlier in the season, insect pollination could be affected). Shifts in rates of growth could thus be especially important for increasing invasive potential since they could result in altered competition with certain members of the community.

The PIP could cause the SCWR to flower earlier or later and for a longer or shorter period of time. Depending on the role flowering of the SCWR plays in the community, it could have either positive or negative effects on the community and surrounding ecosystems (including neighboring crop production). There could be indirect effects (positive or negative) on pollination (and therefore on seed set) of other plants in the SCWR's community or of agricultural crops planted nearby due to changes in the floral display. Simple knowledge of the altered flowering phenology would be insufficient to predict the outcome. Similar reasonable scenarios could be presented for other aspects of phenology, including timing of seed germination, rates of vegetative growth, timing of seed maturation, timing of seed dispersal, etc.

Altered physiology covers such a vast range of potential effects on the SCWR and, therefore, its interactions with other members of the ecological community, it is difficult to say with any precision whether knowledge of physiological changes would aid in assessing impacts of gene flow. Physiological differences play important roles in determining species ranges and the environments within a range that a species inhabits. Some of the physiological changes that might be relevant are changes in photosynthetic rate, changes in water use efficiency, cold tolerance, heat tolerance, and allocation to plant defensive compounds, to name a few. For instance, physiological changes might alter the physiological tolerances of an SCWR which could increase the likelihood of successful colonization of the unoccupied range. Or, in another example, if low water use efficiency had previously kept a given SCWR out of certain dry ecosystems, a PIP for increased efficiency could allow it to colonize dry environments, potentially affecting the composition or dynamics of that community. Drought, cold and salt tolerance traits being currently developed would certainly fall under this category and should be assessed for risks accordingly.

Thus, changes in habit, phenology, and physiology resulting from PIPs could have possible positive or negative effects on seed production and survival, so studies would be needed to assess their potential effects on a case-by-case basis.

Summary:

Although it would be preferable to be able to predict impacts of gene flow on SCWRs with very few pieces of data, in many instances scarce data cannot accurately predict effects of gene flow in complex ecosystems. This is due to the importance of various ecological and evolutionary processes which are inherently system-specific (i.e., specific to the mixture of crop species, SCWR, target organism, and NTOs in a given region). It is those same specifics that ultimately produce a given outcome regarding the potential for a PIP to increase invasiveness or alter population dynamics. The most informative experimental approaches for determining the impact of the PIP involve using PIP-containing SCWR populations from the areas where the PIP crops will be planted and grown in the environments where they naturally occur. Of course, this would likely be too risky due to the potential of PIP escape, so regulatory decisions would have to be made based on data gleaned from studies that employ less realistic environmental conditions and/or community contexts, while also possibly having to mimic the PIP's trait. The less similar the experimental scenario is to the natural scenario found in the field, the more tenuous any conclusions from the data collected would be. This is because many other factors that affect the spread of the PIP may be present in the natural environment. Nevertheless, key experiments could be designed to partially assess the direct and indirect effects of gene flow of PIPs into the SCWR and its ecological community. In Appendix B, the Panel suggests a stepwise approach to the data requirements for determining risk of PIPs' introgression in SCWR populations.

If the impacts of gene flow were judged to be acceptable, based on rigorous experiments, and commercialization were then allowed to proceed, the Panel would recommend monitoring of SCWR populations and the ecological community to (1) determine how well the risk assessment process was able to predict the effects and to (2) catch unanticipated effects as early as possible so mitigation efforts, if needed, could be timely and have the highest possible chance of success. The value of point 1 is that it would provide feedback to EPA on how well the current regulations predict true risk. Where they are functioning well, they could be kept, and where functioning poorly or suboptimally, they could be reevaluated and modified to be more predictive of risk.

Additional comments made by the Panel related to gene flow but that were not responses specific to the charge question are included in Appendix C.

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Appendix A

Publicly Available Databases of Gene and Protein Sequences

Several databases on gene and protein sequences, which are maintained and updated regularly, could serve as a basis for the proposed nomenclature of PIPs. The National Center for Biotechnology Information (NCBI), established in 1988 as a division of the National Library of Medicine (NLM) at the National Institutes of Health (NIH), is widely used by biomedical researchers around the world and has several databases that could be used for that purpose.

(i) GenBank

This database is a collection of publicly available, annotated nucleotide sequences, including mRNA sequences with coding regions, segments of genomic DNA with a single gene or multiple genes, and ribosomal RNA gene clusters. GenBank is an archive of primary sequence data and NCBI does not curate the data. The submitting author has the scientific responsibility for the accuracy of the data. GenBank often contains several entries for the same locus and may include differing sequencing results for a locus due to genetic variations between individuals or organisms or due to sequencing errors. Records can be updated only by the submitting author or an officially designated delegate. Even though data in GenBank are not curated by NCBI personnel, nomenclature in NCBI can be used as a 'de facto standard' because the data bank is in widespread use. Nonetheless, some caution must be exercised since the lack of curation means nomenclatural consistency is not guaranteed. Data are updated daily and have links to other publicly available databases.

Data in GenBank are exchanged daily with other databases, such as the International Nucleotide Sequence Database Collaboration (INSDC) of the European Bioinformatics Institute (EBI) and the DNA Data Bank of Japan (DDBJ).

If the nucleotide sequence in GenBank encodes a protein, the translation product is annotated and a protein accession number (a "protein id") is assigned. This protein id is linked to a record for the protein sequence in NCBI's protein databases.

(ii) RefSeq (Reference Sequence)

This is an alternative database standard in NCBI. It is a curated collection of DNA, RNA, and protein sequences built by NCBI. Several entries in Gen Bank describing the same biological molecule are provided in one entry in RefSeq, removing potential redundancies. However, RefSeq is limited to major organisms for which sufficient data is available.

(iii)**UniProt (Universal Protein Resource)**

UniProt is a protein sequence database that was formed through the merger of three separate protein databases: the Swiss Institute of Bioinformatics' and the European Bioinformatics Institute's **Swiss-Prot**, **TrEMBL** (Translated EMBL Nucleotide Sequence Data Library), and Georgetown University's **PIR-PSD** (Protein Information Resource Protein Sequence Database).

Swiss-Prot and TrEMBL continue as two separate sections of the UniProt database. The Swiss-Prot component consists of manually annotated protein sequence records that have added information, such as binding sites for drugs. The TrEMBL portion consists of computationally analyzed sequence records that are awaiting full manual annotation; following curation, they are transferred to Swiss-Prot.

(iv) **Pfam**

Pfam, another valuable protein resource, is a comprehensive collection of protein domains and families represented as multiple sequence alignments and as profile-hidden Markov models. The current release of Pfam (22.0) contains 9318 protein families. Pfam is based on UniProt and the NCBI GenPept database. Pfam is available on the web from the consortium members in the US on (http://pfam.janelia.org/). Pfam can be searched for novel sequences to find the family of most closely related proteins and can be used in cases where a natural protein product has been extensively modified to achieve the pesticidal function. The annotation in Pfam is based on individual domains and can be used if chimeric protein constructs are part of the PIP.

RNAi databases

(i) **RNAiDB** (http://www.rnai.org/)

This database provides comprehensive access to publicly available RNAi phenotypic data from C. elegans. RNAiDB provides raw data, annotated phenotypes, graphical gene maps, analysis of potential off-target gene inhibition, and tools for searching and mining phenotypic data. However, it is only a useful resource if C.elegans can be used as a model organism for the target species of the pesticide.

(ii) **RNAi Codex** (http://codex.cshl.edu/scripts/newmain.pl)

This RNAi database provides information on RNAi technology for mammalian biology (mouse and human). Investigators can search for shRNA clones by accession number, a keyword, a locuslink ID, gene symbol or unigene cluster identifier. Alternatively, investigators can view clones that appear in functionally linked gene lists that have been hand-curated by experts in the relevant field. Finally a sequence or list of sequences can be used to identify matching shRNAs in the collection.

(iii) **si RNA** (http://sirna.sbc.su.se/)

This database contains information about siRNA molecules from two sources: (i) siRNAs collected from the literature that have experimentally verified efficacy and (ii) siRNAs selected computationally to target the REFSEQ curated human gene set.

Appendix B

Suggested Decision Tree Approach

The Panel's suggestions on a decision tree approach that could be incorporated into the proposed guidelines:

In addition to the direct responsibilities to address the charge questions, the Panel discussed how decisions about gene flow from transgenic plants might be scientifically supported using a decision tree/tiered system of risk assessment. Not all crop x trait releases should require extensive data collection, but some will. It would, therefore, be useful to have a transparent, logical progression that could guide types of expected data collection on a case-by-case basis. Ideally, USDA-APHIS and EPA would unify their data requirements. Below we provide a schema that we hope the EPA will find useful when deliberating the overall structure of regulating PIPs.

The kinds of hypotheses that need testing in relation to gene flow are those that relate to the pertinent ecological and evolutionary processes that could actually inform the exposure x hazard = risk equation. If there are unequivocal results at a given level indicating no risk, further data collection could be terminated. If results were inconclusive or risks appeared possible, the registrant would be asked to continue with subsequent data collection. Experiments involving PIPs should consider at least the first-generation hybrid (F_1) and first-generation backcross (BC_1) between the cultivar and wild species, when biologically plausible. However, these criteria may not be reasonable for long-lived organisms. For some experiments, non-PIP-containing SCWR plants protected from a given target by other means (i.e., pesticide with same activity) could be used. A sample progression might be:

Step 1: Could crop-wild gene flow occur? Determine the potential for hybridization between the wild relatives and the crop possessing the PIP.

A. Investigate the sympatry of the putatively SCWR and the crop. If the SCWR does not co-occur with the crop you can stop. Else go to B.

B. Assess the overlap in crop and wild flowering phenology in the field. If there is no overlap you can stop. Else go to C.

C. Perform crosses in the greenhouse or the field between the potentially SCWR and the crop and determine the number of seeds produced. If no seeds are produced you can stop. Else go to Step 2.

These data would determine whether hybrids can be produced under controlled conditions and whether there is an opportunity for hybridization in the wild. (If there is a chance that the PIP could affect phenology or flowering, then the specifics of the case should be considered here.) If the potential for hybridization is remote or zero, either due to stark differences in flowering phenology, geographic distribution, and/or lack of seed

set/hybrid survival, then there is no need to continue gathering further data. If the potential for hybridization is present then go to Step 2. For some species, including tree species, the potential for hybridization with closely related species is already known in the literature due to the documented presence of hybrids in the wild.

Step 2: Is the pest/disease present in populations of the SCWR? This step would determine whether the SCWR and target pests or NTOs susceptible to the PIP occur sympatrically. This information would partially determine whether selection pressures could act on introgressing PIPs. The problem here is to determine how much data is needed to address the expected variability in patterns of association over time and space. If there is high variability in abundance of target species and susceptible NTOs over time then it would be helpful to define conditions that represent high vs. low risk. For example, if an epidemic occurs every 8 years, yet such events have very strong impact on the populations of the plant species, then they become important events. Nevertheless, it may be difficult to adequately represent such variation in target species found in populations of the SCWR. If the target pests or beneficial NTOs do co-occur, in at least some populations some of the time, then go to Step 3. If not, stop here. In many circumstances it may be easier to estimate a fitness impact under controlled conditions than to rigorously determine the distribution of the target species in the SCWR populations; therefore, Step 3 can be done before Step 2. If Step 3 is negative, one could stop and would not need to gather data on step 2.

Step 3: Does introgression of the transgene have fitness consequences for the SCWR such that the PIP would be expected to increase in frequency due to selection? Assess the fitness of PIP and non-PIP containing (or pest protected and non-protected) wild plants in the presence and absence of the target species. Fitness measures ideally include lifetime survival and seed production. Such measures are easier to obtain for annual plants, but can be quite difficult to measure in long-lived perennials. For the latter, fitness of the plant is often estimated over one or two years. Indirect measures are also used, including estimates of flower production, rather than seed set, or for particularly long-lived species using plant size as a surrogate for fitness. The less direct the measure of fitness, the less accurate the estimate of relative fitness of transgenic and non-transgenic wild plants. If carrying a PIP provides a selective advantage in the presence of the target species, then the PIP gene is expected to increase in frequency in the population. If the PIP produces a fitness cost in the absence of the target, the PIP would be selected against when there are no infestations. If the PIP does not provide a selective effect in the presence of the target species, go to Step 4 to ensure that other factors do not maintain PIP in the wild species. If the PIP does provide a selective advantage or a strong cost, you should gather data for Steps 5 and 6. (Note: Given that it will often be easier and more practical to determine a fitness impact under controlled conditions than rigorously determining effects of the PIP on target species in geographically appropriate SCWR populations, this was chosen as Step 3, but Step 6 could also be done first.)

Step 4: Could recurrent gene flow into a SCWR population or genetic drift in SCWR populations result in increased frequencies of the PIP in some of the populations? Data on the mode of pollination, breeding systems of the SCWR and the

crop, likely distance of SCWR populations to crop fields, and likely crop rotations should be used to determine whether gene flow from the crop to the SCWR populations could be high. Similarly, in SCWRs where small populations form a metapopulation, genetic drift could fix the PIP allele in some populations, while it would be lost in other populations. This is important even in the absence of selective advantage because either recurrent, heavy gene flow or genetic drift are alternative processes that could maintain PIPs at high frequency, thereby potentially affecting population dynamics. If there is low potential for gene flow and genetic drift, and no selective advantages have been demonstrated in Step 3, stop here if one is confident that the PIP does not directly affect NTOs. Else, go to Step 5.

Step 5: Are there non-target organisms associated with the SCWR in wild populations? NTOs can be susceptible or resistant to the PIP and have beneficial, neutral, or detrimental effects on SCWR populations. It is thus important to determine the nature and distribution of NTOs that play a role in regulation of SCWR populations. Such organisms could alter the impact of a PIP on SCWR populations.

Determine whether NTOs are present and whether they appear to affect the SCWR plants under natural conditions. If susceptible NTOs are present and they negatively affect the SCWR, then, SCWR fitness may increase in the presence of the PIP. Similarly, if the NTO has a positive effect on fitness and is susceptible to the PIP, then SCWR fitness could be reduced in the presence of the PIP. NTOs that are resistant to the PIP will not be affected by PIP presence. However, resistant NTOs with negative effects on the SCWR could become even more important regulators of SCWRs population dynamics if susceptible target pests and NTOs are eliminated. Identifying NTOs helps determine whether the selective advantage of the PIP is likely to vary under natural conditions relative to more controlled conditions (greenhouse for e.g.). Because factors besides NTOs may also influence the target species and its impact on the wild species under natural conditions, it is best to always go to Step 6 from Step 5 whether or not NTOs have been identified.

Step 6: Is there a selective advantage to the PIP under field conditions? Although there may be a strong effect of the target species on the SCWR in the greenhouse, other factors in the field may reduce such an impact. Such factors include the low frequencies of the target species in wild SCWR populations and presence of NTOs, as well as abiotic and biotic interactions that could strongly influence the fitness of SCWR under field conditions. These experiments would help determine the impact of the PIP on the fitness of the SCWR under field conditions. Such impact could be hypothesized by responses to parts 2, 3 and 5 and the experiments described below could be viewed as testing such predictions. Alternatively, if testing PIP under field conditions is not possible, information gathered in Step 5 can be combined with the fitness data collected in Step 3 in order to build mathematical models of the system (population genetic-type models) and improve our predictions of the impact of the PIP on the wild species and the community.

The impact of the PIP on SCWR in natural populations depends on the abundance of the target species over time and space and on the effect of the target species on the fitness of the SCWR. At the same time, it depends on the impact of the PIP on NTOs and their impact on the fitness of the SCWR and other host species in the community. Knowing the strength of selection on the PIP in naturally occurring SCWR populations would help determine the likelihood that the PIP would increase in frequency in populations of the SCWR over time. If the target species appears to strongly regulate the SCWR populations, then we might expect PIP introgression and, as a consequence, population dynamics may change, noted by an increase in λ, and a potential for the SCWR to become invasive. If there is no significant selective advantage of carrying the PIP in naturally occurring SCWRs, then stop. Else, go to Step 7 or do not commercialize.

Step 7: Does the PIP increase population growth (λ) by altering demography and/or population dynamics? Perform a demographic experiment to determine the impact of the transgene on λ (population growth rate) to address the potential impact of the PIP on SCWR population dynamics. Experiments that compare λ between populations of PIP and non-PIP containing plants, in the presence and absence of the target pests and NTOs, would be performed. For non-annual SCWRs, estimate λ over a few years to determine its variability and use such variability in models of population growth (matrix models) for the plant species over time. Measurements of λ can originally be done under greenhouse conditions or highly controlled environments as was done for fitness measurements in Step 3. However, these experiments should ultimately be conducted, if possible, in natural conditions as was done for fitness measures in Step 6. If there is no change in λ, for PIP-containing SCWRs, commercialization may be viable. If λ increases or decreases greatly, we reach a point of a high risk for increased invasive potential or species loss, respectively.

A final step would be to test if the PIP could increase the ability of the SCWR to spread to or colonize unoccupied areas. However, the experiments would be difficult to design and carry out and strong evidence for interpretation of results may not exist. Therefore, Step 7 would be the final step.

To learn whether this decision-tree successfully eliminates risky PIPs, long-term post-release monitoring of PIPs judged to be safe could inform future releases or alert for the need to eradicate. Of course, initial studies of new crop-wild complexes will be more onerous in terms of data collection than studies of later events. Studies of new traits will also be more difficult. Plus, risk assessment for long lived perennials with higher pollen/seed production over more years remains difficult, although matrix modeling using λ calculated over multiple years could be of use.

Appendix C

Additional Panel Comments on Gene Flow

Ecological versus ecotoxicological approaches. During the public comments, many individuals discussed a need to use an ecotoxicological approach when doing risk assessment. Ecotoxicology was presented as a rigorous scientific approach that involves careful problem formulation for risk assessment, testing of (null) hypotheses, and presentation of, thus, strong data that is useful for risk assessment. Some of the same presenters contrasted this with the ecological approach, which they presented as somewhat directionless, not hypothesis driven, and, therefore, of little use for risk assessment. We will discuss these approaches briefly and clarify that this is a false dichotomy that will not improve risk assessment.

Ecotoxicology is defined by some as the study of "ecology in the presence of toxicants" (Chapman 2002) in that it aims to assess the impacts of toxins on many levels, from the level of the cell to that of the ecosystem (Maltby & Naylor 1990). In a 2007 paper heavily cited in public comment, Raybould describes ecotoxicology as being driven by policy-required risk assessment, using null hypotheses of no harm, which are rigorously tested to produce data with the strong ability to predict effects. He contrasts ecotoxicology with ecology, which he presents as having solely scientific goals, which build on one another, sometimes resulting in complex predictions, tested realistically and resulting in data that is not necessarily helpful for risk assessment.

Two members of the Panel commented that setting the ecological vs. ecotoxicological approaches against one another represents a false dichotomy. All of the scientific approaches discussed here aim to generate strong data from testable hypotheses directly applicable to risk assessment. Gene flow itself, and any subsequent changes in PIP transgene frequency, are inherently topics studied by evolutionary ecology and are thus best studied by testing ecologically or evolutionarily relevant hypotheses regarding what are necessarily complex responses. From public comments made on the above dichotomy, these two Panel members are concerned that the attempt to simplify and narrow hypotheses (as seemed to be the goal of proponents of the ecotoxicological approach as applied to risk assessment for genetically modified crops) to address only very few predetermined questions will be more likely than an ecological approach to produce research that is unable to warn against risky PIP products. Previous experience with invasive species tells us that studying fundamental ecological interactions is important for having any hopes of predicting future detrimental effects. In a risk assessment framework, there will be times when difficult or time-consuming ecological studies will be worthwhile and necessary before allowing commercialization to proceed.

Guidelines should not impede production of economically or ecologically important PIPs. Concerns were expressed by one Panel member that the guidelines related to gene flow between PIP plants and SCWRs could impede and potentially stop the production of PIPs of great ecological and/or economic importance. Examples of plants that could benefit from PIP technology include the American Chestnut, *Castanea*

dentata, once a common tree in Eastern North American hardwood forests that has been devastated by Asian bark fungus *Cryphonectria parasitica* (a.k.a., chestnut blight), and citrus trees that are vulnerable to a bacterial disease called citrus greening *(Liberobacter* spp.). Citrus greening has no known cure and threatens the citrus industry wherever it occurs. Current regulatory requirements also inhibit scientists from developing technologies that are required to produce some PIP plants, especially methods needed to produce PIP trees, ornamentals and other perennial plants. The Panel member recommended that the EPA should provide avenues for discussion for interested scientists so that on a case-by-case basis risk/benefit assessments could be made along with appropriate problem formulation to identify concerns, uncertainties and an analytical plan to address needs. A roadblock, perhaps unintentional, that potential registrants now face could be considered a "catch 22" because PIP plants are required to make environmental assessments, but PIP plants cannot be produced, at least economically, because of the requirements for environmental assessments. The Panel member also noted the different standards for judging risks for PIPs compared with traditional breeding methods. For example, a transgenic tree theoretically could have one gene that could confer resistance to a pathogen, whereas a hybrid tree often contains hundreds, if not thousands, of new genes of which only a few are related to pathogen resistance. In most cases little is known about the other genes, especially how they might impact the environment.

Defining harm. One Panel member remarked that it was not science's job to determine what constitutes "harm" to wild populations and ecosystems. Instead, that it is a societal value judgement. Another Panel member indicated that increase in λ could be considered harm and this criterion could hold for at least all disease and insect resistance PIPs.