MEETING DOCUMENT: FIFRA SAP MEETING CONCERNING PLANT-INCORPORATED PROTECTANTS BASED ON PLANT VIRAL COAT PROTEIN GENES (PVCP-PIPS)

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Purpose

The purpose of this meeting is to seek the advice of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) on a number of technical issues associated with a type of pesticide, plant-incorporated protectants based on plant virus coat protein genes (PVCP-PIPs). To this end, the Environmental Protection Agency (EPA) is providing to the FIFRA SAP this cover document which poses a series of questions to the SAP, and four attached documents, which provide additional information on the topics before the Panel. The four attachments are:

Attachment I: Draft Approach to Exempting Certain PVCP-PIPs from Regulation under FIFRA

Attachment II: Draft Approach to Exempting Certain PVC-Proteins from the Requirement of a Tolerance under FFDCA

Attachment III: Environmental Risk Assessment of Plant Incorporated Protectant (PIP) Inert Ingredients

Attachment IV: Minutes of the October 13-15, 2004 FIFRA Scientific Advisory Panel Meeting on Issues Associated with Deployment of a Type of Plant-Incorporated Protectant (PIP), Specifically those Based on Plant Viral Coat Proteins (PVCP-PIPs)

The material introducing each question articulated in this cover document gives a general cursory overview of the issue on which EPA requests technical advice from the SAP. For additional information on these questions, SAP members are directed to the relevant sections of first three attachments which provide more detailed descriptions of the Agency analysis of the technical issue. In some questions, the Agency asks the SAP whether Agency interpretations of available information could support the technical rationales advanced by the EPA in these attachments.

As this meeting builds on a previous meeting of the SAP on PVCP-PIPs, EPA includes in this package the minutes of the October 13-15, 2004 meeting as the fourth attachment to this cover document.

Introduction

A plant-incorporated protectant (PIP) is defined as a pesticidal substance that is intended to be produced and used in a living plant, or in the produce thereof[1], and the genetic material necessary for production of such a pesticidal substance. The definition includes both active and inert ingredients[2].

PIPs may be genetically engineered into plants[3]. Some specific genetic sequences, when incorporated into a plant's genome, can endow the plant with the ability to resist damage from certain pests. EPA considers plant virus coat protein PIPs (PVCP-PIPs) to be those PIPs based on one or more genes that encode a coat protein of a virus that naturally infects plants. *This includes PVCP-PIPs that produce no protein*. Incorporation of plant viral coat protein gene sequences into plant genomes has been found to confer resistance to the virus from which it was derived, and often to related viruses (OECD Environment Directorate 1996).

PVCP-PIPs are regulated as pesticides by EPA under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) because they meet the FIFRA definition of a pesticide, being intended for preventing, destroying, repelling, or mitigating a pest. Residues of PVCP-PIPs in food are regulated by EPA under the Federal Food, Drug, and Cosmetic Act (FFDCA).

Background – FIFRA Regulation

In 1994, EPA proposed two options to exempt PVCP-PIPs under FIFRA. The first option was a full categorical exemption based on the rationale that PVCP-PIPs generally pose a low probability of risk to human health and the environment. However, recognizing that other plants could acquire the virus resistance through hybridization with a transgenic plant, an alternative to a full categorical exemption was also proposed. Under this alternative exemption option, the Agency defined a set of criteria to identify those PVCP-PIP/plant combinations with the lowest potential to confer selective advantage on wild or weedy plant relatives. Only those PVCP-PIPs that met the criteria would have been exempt from regulation.

EPA has yet to finalize a FIFRA exemption for PVCP-PIPs, in part because more recent information has raised questions about whether all PVCP-PIPs pose low risks, and EPA must be able to make such a finding in order to exempt all PVCP-PIPs under FIFRA. For example, the 2000 National Research Council (NRC) report, Genetically Modified Pest-Protected Plants, recommended that

[1] The phrase "or produce thereof" is included in the definition of a PIP to make it clear that pesticidal substances active in the fruit or other plant product for pesticidal purposes are also considered to be PIPs.
[2] "Inert ingredient" means any substance, such as a selectable marker, other than the active ingredient, where the substance is used to confirm or ensure the presence of the active ingredient, and includes the genetic material necessary for the production of the substance, provided that genetic material is intentionally introduced into a living plant in addition to the active ingredient.
[3] PIPs may also be found naturally occurring in plants or may be introduced through conventional breeding. However, the focus here is on PIPs based on plant viral coat protein genes that are introduced into plants through genetic engineering.

> "EPA should not categorically exempt viral coat proteins from regulation under FIFRA. Rather, EPA should adopt an approach, such as the agency's alternative proposal, that allows the agency to consider the gene transfer risks associated with the introduction of viral coat proteins to plants."

In addition to the risks associated with gene transfer, the 2000 NRC report considered other risk issues in the context of PVCP-PIPs, including the potential for adverse effects following recombination, heterologous encapsidation, and synergy. Although the report concluded that, "[m]ost virus-derived resistance genes are unlikely to present unusual or unmanageable problems that differ from those associated with traditional breeding for virus resistance," it also suggested strategies to reduce or eliminate such concerns. Neither formulation of the exemption EPA proposed in 1994 contained provisions that would have enabled EPA to ensure that the risk management strategies suggested by the NRC would be implemented, e.g., elimination of specific sequences to limit the potential for recombination.

Background – FFDCA Regulation

In 1994 EPA proposed exempting the plant virus coat protein portion of a PVCP-PIP (PVC-protein) from the requirement of a food tolerance under FFDCA based on the rationale that (1) virus infected plants have always been a part of the human and domestic animal food supply and (2) plant viruses have never been shown to be infectious to humans or other mammals. The safety of consuming plant virus genes has since been supported by experimental investigations (Chen et al. 2003; Rogan et al. 2000; Shinmoto et al. 1995) and expert consultations including the 2000 NRC report which concluded that, "viral coat proteins in transgenic pest-protected plants are not expected to jeopardize human health because consumers already ingest these compounds in nontransgenic food" (National Research Council 2000).

EPA has not finalized the proposed tolerance exemption for PVC-proteins[4], in part because it has been unclear how to describe the PVC-proteins that have a history of safe human dietary consumption and that would therefore fall within the base of information supporting the 1994 proposal. EPA recognizes that PVCP-PIP developers may need to modify the coat protein gene for appropriate gene expression or may wish to modify the coat protein gene to achieve other goals, e.g., to reduce the frequency of recombination. Such modifications might result in changes to the protein produced such that the rationale used to support exemption (i.e., a history of exposure) might not apply.

EPA's Current Approach

In October 2004, EPA consulted the FIFRA SAP on a number of scientific issues identified for PVCP-PIPs. After carefully considering this advice and other available scientific and regulatory

[4] Although a general tolerance exemption covering a category of PVC-proteins has not been finalized, tolerance exemptions for specific PVC-proteins have been issued (i.e., coat protein of Potato Virus Y, coat protein of Watermelon Mosaic Virus-2 and Zucchini Yellow Mosaic Virus, coat protein of Papaya Ringspot Virus, and coat protein of Cucumber Mosaic Virus). Nucleic acids are also currently exempt from FFDCA tolerance requirements (40 CFR 174.475).

information, the Agency is now developing proposals for an exemption under FIFRA and an exemption under FFDCA.

Under FIFRA, one approach that EPA is considering would involve criteria that would clearly identify and exempt only those PVCP-PIPs that fall within the base of experience used to support the exemption and pose low risk to human health and the environment. Under this approach, EPA has identified three possible criteria, each of which could be satisfied in one of two ways. The first method, articulated in paragraph (1), describes an objective, well-defined characteristic of the PVCP-PIP and could therefore be self-evaluated by a product developer. The second method, described in paragraph (2), involves consideration of several types of information, and an Agency review would therefore be required to determine qualification. PVCP-PIPs that do not qualify for any proposed FIFRA exemption could be submitted for a case-by-case review for registration as is required for non-exempted PIPs distributed or sold in commerce.

Under FFDCA, EPA is considering two possible tolerance exemptions for PVC-protein residues: a categorical exemption for a subset of PVC-proteins based on objective, well-defined characteristics of the PVC-protein, and an exemption conditional on an Agency determination after review that certain other criteria are met. The potential criteria in both exemptions are intended to identify clearly only those residues for which a long history of safe exposure and consumption can support exemption. PVC-proteins that qualify for neither proposed tolerance exemption could be submitted for a case-by-case review for an individual tolerance exemption as is required for all non-exempted PIP residues that may be present in food or feed.

The FIFRA SAP is being asked to advise the Agency on a number of scientific issues pertinent to determining the potential impact of these approaches on human health and the environment. Four relevant documents are attached.

Attachment I, “Draft Approach to Exempting Certain PVCP-PIPs from regulation under FIFRA,” outlines the scientific issues EPA has considered in evaluating PVCP-PIPs for possible exemption.

Attachment II, “Draft Approach to Exempting Certain PVC-Proteins from the Requirement of a Tolerance under FFDCA,” outlines the scientific issues EPA has considered in evaluating PVC-proteins for a possible tolerance exemption.

Attachment III, “Environmental Risk Assessment of Plant Incorporated Protectant (PIP) Inert Ingredients,” covers EPA’s environmental risk assessment of six selectable markers.

Attachment IV, “Minutes of the October 13-15, 2004 FIFRA Scientific Advisory Panel Meeting on Issues Associated with Deployment of a Type of Plant-Incorporated Protectant (PIP), Specifically those Based on Plant Viral Coat Proteins (PVCP-PIPs),” is the minutes of the most recent SAP meeting convened to address issues associated with PVCP-PIPs.

Charge Questions to the Panel

Gene Flow Issues

The first criterion EPA is considering under any proposed FIFRA exemption concerns gene flow/transfer. While gene flow from plants containing PVCP-PIPs does not necessarily constitute an environmental risk, there are concerns that wild or weedy relatives that could acquire the PVCP-PIP might acquire the potential to escape any significant growth and/or reproduction constraints imposed on the plant by natural virus infection, and such events could in turn impact the ecosystem to a degree that is not yet predictable.

Although many events must occur before the transfer of a PVCP-PIP from crop plants to wild or weedy relatives would significantly change plant population dynamics, such a series of events could reasonably be expected to occur for a few PVCP-PIP/plant combinations. Research showing that plants infected with viruses often have decreased growth, survivorship, and/or reproduction (Yahara & Oyama 1993; Friess & Maillet 1996; Funayama et al. 1997; Maskell et al. 1999) suggests that introgression of a virus resistance gene into some plant populations might allow a population to outcompete other plant species (Power 2002) or have other effects on ecosystem relationships. In addition, a few studies confirm that virus infection can in some cases affect plant population dynamics (Jones & Nicholas 1998; Funayama et al. 2001). Acquisition of virus resistance has also been found in some cases to decrease plant fitness (e.g., Remold 2002), due in one case to plants becoming more attractive to herbivores when not infected by viruses (Gibbs 1980). Such considerations may be important in evaluating effects on endangered/threatened species. Whether these types of changes could result in adverse environmental effects is still unresolved.

In order to develop a proposed exemption, EPA seeks a straightforward, easy-to-understand criterion to identify those crop plants containing a PVCP-PIP that pose low probability of risk with respect to the potential of gene transfer to lead to significant changes in plant population dynamics. The inability of the plant to form viable hybrids with wild or weedy plants in the United States provides straightforward assurance in a clearly articulated criterion that adverse effects due to gene transfer are unlikely to occur for a particular PVCP-PIP/plant combination.

With the assistance of the October 2004 SAP, EPA has identified the following plants as not having wild or weedy relatives in the United States, its possessions, or territories[5] with which they can produce viable hybrids in nature: almond (*Prunus communis*), apricot (*Prunus armeniaca*), asparagus (*Asparagus officinale*), avocado (*Persea americana*), banana (*Musa acuminata*), barley (*Hordeum vulgare*), bean (*Phaseolus vulgaris*), black-eyed pea (*Vigna unguiculata*), cacao (*Theobroma cacao*), celery (*Apium graveolens*), chickpea (*Cicer arietinum*), citrus (*Citrus spp.*), coffee (*Coffea arabicua*), corn (*Zea maize*), cucumber (*Cucumis sativus*), eggplant (*Solanum melongena*), guava (*Psidium guajava*), kiwi (*Actinidia spp.*), mango (*Mangifera indica*), nectarine (*Prunus persica*), okra (*Abelmoschus esculentus*), olive (*Olea europaea*), papaya (*Carica papaya*), parsley (*Petroselinum crispum*), pea (*Pisum sativum*), peach (*Prunus persica*), peanut (*Arachis hypogaea*), pineapple (*Ananas comosus*), pistachio (*Pistacia vera*), plum (*Prunus domestica*), potato (*Solanum tuberosum*), soybean (*Glycine max*), spinach

[5] Includes the Commonwealth of Puerto Rico, the Virgin Islands, Guam, the Trust Territory of the Pacific Islands, and American Samoa.

(*Spinacia oleracea*), starfruit (*Averrhoa carambola*), taro (*Colocasia esculenta*), tomato (*Lycopersicon lycopersicum*), or watermelon (*Citrullus lanatus*).

1(a). **Does this list identify plant species that would present low risk of conferring any selective advantage on a wild or weedy relative in the United States, its possessions, or territories[5] were they to contain a PVCP-PIP? Please explain the basis for your answer, providing documentation to support your decision.**

The October 2004 SAP noted that some of the plants on this list (i.e., asparagus and celery) were able to escape cultivation and form occasional volunteer populations. EPA notes that in addition to these two species, many other species from this list have naturalized populations in the United States (i.e., plants occurring in natural areas outside of agricultural fields and not simply volunteer plants within other fields). For example, the USDA PLANTS database (accessible at http://plants.usda.gov/) indicates that almond, apricot, avocado, banana, barley, bean, black-eyed pea, cacao, chickpea, citrus, coffee, corn, cucumber, eggplant, guava, mango, okra, olive, papaya, parsley, pea, peach, peanut, pineapple, plum, potato, soybean, spinach, taro, tomato, and watermelon have "native or naturalized"[6] populations in the United States.

Based on this information, it appears that the ability to naturalize is common for crops. However, it can be hypothesized that naturalized populations of these particular crop plants possess a suite of traits that facilitate cultivation in a managed habitat and likely confer a selective disadvantage on plants in the wild. Acquisition of a single trait, i.e., virus resistance, would therefore not be expected to provide sufficient competitive advantage to make naturalized populations of these plants significant weed problems outside of agricultural fields.

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

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

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[6] In the PLANTS database, Native means naturally occurring in North America and the U.S. and its territories at the time of Columbus; Introduced means arrival from some other part of the world since Columbus's time. Naturalized plants are introduced plants that now exist in the wild without assistance from humankind; in PLANTS these are called Introduced since this term is more familiar to most people than naturalized. In PLANTS, "Native and Introduced" applies to species with both native and introduced varieties or subspecies. And if a plant is native to one part of the U.S. and introduced in another, it is coded as Native.

A list of plants such as those in question 1 offers a well-defined criterion for identifying crop plants that pose low probability of risk with respect to concerns associated with gene transfer when they contain a PVCP-PIP. However, EPA recognizes that other, perhaps less well-defined criteria could also identify PVCP-PIPs that are low risk. EPA is requesting the SAP's advice on whether a PVCP-PIP would pose low risk with respect to concerns associated with gene flow if the Agency determines that the plant containing the PVCP-PIP (i) is itself not a weedy or invasive species outside of agricultural fields in the United States, its possessions, or territories, and (ii) does not have relatives outside of agricultural fields in the United States, its possessions, or territories that are weedy or invasive species or endangered/threatened species with which it can produce viable hybrids in nature.

The rationale for such a criterion would be that there is a low probability that acquisition of a virus-resistance trait would confer on plants that are not already weedy or invasive sufficient additional competitive advantage to lead to adverse environmental outcomes. With regard to consideration of whether a plant population is under viral disease selection pressure and whether this pressure is the only condition restraining the population, conventional agriculture offers some insight. Virus-resistant varieties of certain crops have been bred and grown in the past, generally using a wild relative of the crop plant as the source of resistance. There is no indication that growing such crop plants near wild or weedy relatives results in these relatives becoming any more of a weed problem due to acquiring virus resistance from the crop (National Research Council 1989). It is unlikely that use of PVCP-PIPs would affect wild or weedy relatives differently than virus resistant varieties developed through traditional breeding have affected wild or weedy relatives in the past. In addition, outbreeding depression between crop plants and their wild relatives appears to be more common than hybrid vigor (Hails & Morley 2005). In outbreeding depression, mating between individuals from two different environments can disrupt gene combinations that are favored by natural selection in each environment. Resulting offspring may have phenotypes that are poorly adapted to the habitat of either parent. Thus, hybrid offspring acquiring a PVCP-PIP are often likely to be less competitive than their wild parent in nature. When EPA asked the FIFRA SAP in 2004 about the likelihood that plant populations freed from viral pressure could have increased competitive ability leading to changes in plant population dynamics, the FIFRA SAP offered the following opinion: "[b]ased on knowledge obtained from observation of cultivated crops in the agroecosystem, the majority of the [2004] Panel concluded that it would be unlikely that a plant population freed from viral pressure would give a plant species a competitive advantage" (Ref. U.S. Environmental Protection Agency 2004).

2(a). **Please comment on whether the following criteria would allow the Agency to identify correctly those PVCP-PIPs that present low risk with respect to environmental concerns associated with gene flow of a PVCP-PIP. What data supports or refutes the Agency's rationale for developing these criteria?**

(i). **the plant containing the PVCP-PIP is itself not a weedy or invasive species outside of agricultural fields in the United States, its possessions, or territories, *and***

(ii). **the plant containing the PVCP-PIP does not have relatives outside of agricultural fields in the United States, its possessions, or territories that are weedy or invasive species or endangered/threatened species with which it can produce viable hybrids in nature.**

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

In its evaluation of (i) and (ii) above, EPA would consider the most recent scientific information about the plant species containing the PVCP-PIP and its wild or weedy relatives to evaluate the potential for weedy or invasive behavior, including whether any of these species are extending their range. The Agency would evaluate a number of sources including existing lists of invasive weeds, e.g., the Federal Noxious Weed List. Inclusion on any given list would be informative, but not determinative for the Agency's evaluation. Examination of existing lists has shown that different organizations use different criteria for listing species depending on the goals and missions of those organizations. Thus, the Agency is considering using existing lists as a resource much as it would use published literature, rather than as determinative sources. For example, plants that may form volunteer populations in agricultural fields are considered weeds by some organizations and may appear on those organizations' weed lists, but EPA would not consider propensity to volunteer, i.e., to grow in a field from seeds dropped from the previous crop rotation, to be indicative of general weediness potential for a plant.

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

Viral Interactions Issues

The second criterion EPA is considering as part of a FIFRA exemption concerns viral interactions. The Ecological Society of America noted that such interactions could lead to the creation of viruses with enhanced disease characteristics or new transmission properties (Snow et al. 2005). However, mixed viral infections are extremely common in crops and other plants (Hammond et al. 1999). In natural, mixed infections, viral genomes from different strains and/or different species simultaneously infect the same plant and thus have opportunities to interact (e.g., through recombination, heterologous encapsidation, or synergy). In spite of many opportunities for interaction in nature, such events rarely lead to any detectable adverse outcome (Falk & Bruening 1994). However, such *in planta* interactions do have the potential to result in a virus that causes increased agricultural or other environmental damage. For example, numerous recombination events among tomato-infecting begomoviruses around the Nile and Mediterranean Basins are thought to be at least partially responsible for numerous whitefly-transmitted tomato diseases that have emerged in the last 20 years (Fauquet et al. 2005). In

addition, as the 2004 SAP pointed out, “[i]n contrast to heterologous encapsidation and synergy, at least in theory, the impact of recombination could be much greater, since there is no abundant bioinformatics evidence that recombination has indeed, as had been long suspected, played a key role in the emergence of new viruses over evolutionary time” (U.S. Environmental Protection Agency 2004). Interactions between viral transgenes and an infecting virus may be a concern to the extent that such events are novel, i.e., involve viruses that would otherwise not be expected to interact in a mixed infection found in nature.

The Agency asked the FIFRA SAP during the October 2004 meeting to what extent PVCP-PIPs in plants might present a potential concern should interactions with infecting viruses occur. The Panel expressed concern only “about certain limited situations” and stated that “except perhaps for a very few cases, neither heterologous encapsidation nor synergy should be considered to be of serious concern” and "in most cases there is little a priori reason to believe that recombinants between viruses and transgenes will be more of a problem than recombinants between two viruses infecting the same plant, unless transgenes are derived from severe or exotic isolates. The general recommendation to use mild, endemic isolates as the source of the transgene (e.g. Hammond et al. 1999) should minimize any potential for creation of novel isolates that would not equally easily arise in natural mixed infections."

Based on the advice from the October 2004 SAP meeting, EPA has developed the following language to identify PVCP-PIPs that present low risk with respect to concerns associated with viral interactions (i.e., recombination): the viral pathotype[7] used to create the PVCP-PIP has naturally infected[8] plants in the United States, its possessions, or territories and naturally infects plants of the same species as those containing the PVCP-PIP.

The rationale for such a criterion would be that if the viral pathotype meets these conditions, the recombinants that could be produced in that plant would, in principle, be no different than what could occur in a natural mixed infection in the United States involving that virus. Mixed virus infections occur frequently in nature (Hammond et al. 1999) and thus provide numerous opportunities for viruses in the United States that infect the same plant species to interact. EPA seeks to identify those situations that clearly pose low risk with respect to viral interactions, i.e., those situations in which recombination in a transgenic plant would involve segments of viruses that already have the opportunity to recombine in a natural, mixed infection.

[7] EPA uses the term “viral pathotype” rather than the more generic term “virus” in response to the FIFRA SAP comment in October 2004, that “[n]ot all isolates of a virus infect and cause disease in all plant genotypes and, as a consequence, the unqualified use of the term “virus” when setting a condition for applicants to the Agency [is] not adequate in this context. It is therefore appropriate in the context of biosafety as well as virus epidemiology to recognize the value of defining specific viral pathotypes or host range variants.”

[8] EPA means by the term “naturally infect” to infect by transmission to a plant through direct plant-to-plant contact (e.g., pollen or seed), an inanimate object (e.g., farm machinery), or vector (e.g., arthropod, nematode, or fungus). It does not include infection by transmission that occurs only through intentional human intervention. The Agency wants specifically to exclude transmission that occurs only through intentional human intervention, e.g., manual infection in a laboratory or greenhouse setting, because such transmission would have little relevance to normal human dietary exposure. EPA intends to include viruses that are likely to have been part of the human diet due to their ability to spread without intentional human intervention. EPA recognizes that humans may play an inadvertent role in infection (e.g., by transmitting the virus on farm machinery). Such unintentional (and often unavoidable) transmission can be an important means of virus transmission, and this mode of transmission would be included under “naturally infects.”

3. **Please comment on the usefulness of the following criteria (i) and (ii) in correctly identifying PVCP-PIPs that present low risk with respect to environmental concerns associated with novel viral interactions[9]. Please explain the basis for your answer, including whether the limitations imposed by the use of "viral pathotype,"[7] "naturally infect,"[8] "species," and "United States, its possessions, or territories" are necessary and/or sufficient. For example, could other parts of North America be included as part of criterion (i)?**

   (i) **the viral pathotype used to create the PVCP-PIP has naturally infected plants in the United States, its possessions, or territories and**

   (ii) **the viral pathotype used to create the PVCP-PIP naturally infects plants of the same species as that containing the PVCP-PIP.**

Other characteristics of the PVCP-PIP may also indicate low risk, even though criteria describing such characteristics cannot be as clearly articulated as those described by the language in question 3. EPA is requesting the SAP's advice on whether a PVCP-PIP would pose low risk with respect to viral interactions if the Agency determines that (i) the properties of the viral pathotype that are determined by the coat protein gene used to create the PVCP-PIP are substantially similar to the properties of a viral pathotype that naturally infects plants in the United States, its possessions, or territories, and the viral pathotype used to create the PVCP-PIP naturally infects plants of the same species as that containing the PVCP-PIP, or (ii) viruses that naturally infect the plant containing the PVCP-PIP are unlikely to acquire the coat protein sequence through recombination and produce a viable virus with significantly different properties than either parent virus.

The rationale supporting criterion (i) is that if the properties of the viral pathotype that are determined by the coat protein gene are substantially similar to the properties of a viral pathotype that naturally infects plants in the United States and the viral pathotype used to create the PVCP-PIP naturally infects plants of the same species as those containing the PVCP-PIP, the viral interactions that could occur in a plant containing such a PVCP-PIP would be no different than what could occur in a natural mixed infection in the United States involving that virus. To evaluate this criterion, EPA would consider first the sequence similarity in the coat protein gene of the pathotype used and pathotypes found in the United States. Then, when information is available, EPA would evaluate the extent to which any significant deviations from pathotypes in the United States are likely to influence phenotypic properties of the virus. The rationale supporting criterion (ii) is that even if criterion (i) is not met, it may be possible to reduce the frequency of recombination to such an extent that few viral interactions, novel or not, are expected to occur. Recombination is the sole type of viral interaction focused upon based in part on conclusions of the 2004 SAP that "except perhaps for a very few cases, neither heterologous encapsidation nor synergy should be considered to be of serious concern" (U.S. Environmental Protection Agency 2004).

[9] A "novel viral interaction" is an interaction (i.e., recombination, heterologous encapsidation, or synergy) between viral transgenes and an infecting virus involving viruses that would otherwise not be expected to interact in a mixed infection found in nature.

4(a). **Please comment on the usefulness of the following criteria (i) and (ii) in allowing the Agency in its review of the product to identify correctly whether the PVCP-PIP presents low risk with respect to environmental concerns associated with novel viral interactions[9]. Please explain the basis for your answer.**

(i) the properties of the viral pathotype that are determined by the coat protein gene used to create the PVCP-PIP are substantially similar to the properties of a viral pathotype that naturally infects plants in the United States, its possessions, or territories, and the viral pathotype used to create the PVCP-PIP naturally infects plants of the same species as that containing the PVCP-PIP, or

(ii) viruses that naturally infect the plant containing the PVCP-PIP are unlikely to acquire the coat protein sequence through recombination and produce a viable virus with significantly different properties than either parent virus.

The Agency review of (i) could involve a consideration of data from a number of different sources including virus coat protein sequence data from public repositories and developer-generated data on the natural range of variation of coat protein genes for particular viral pathotypes. In review of (ii), the Agency might consider (a) if the PVCP-PIP confers virus resistance through post-transcriptional gene silencing thereby greatly reducing the amount of RNA available for recombination, (b) if the PVCP-PIP construct is designed to reduce the frequency of recombination (e.g., Miller 2000; Nagy & Bujarski 1996; Nagy & Bujarski 1998; Nagy et al. 1999; Teycheney et al. 2000), or (c) if the inserted coat protein sequence is only a relatively small portion of the naturally occurring sequence suggesting that viruses acquiring the region are unlikely to acquire a novel phenotype. EPA recognizes the comments of the 2004 SAP that "methods for minimizing recombination are only partially effective. For this reason, the question remains whether novel recombinants would be created in transgenic plants, and simply reducing the frequency of these events is not an answer to the question" (U.S. Environmental Protection Agency 2004). However, a combination of two or more methods, or even perhaps a single method in some cases, might reduce the expected frequency of recombination such that a PVCP-PIP would pose low risk with respect to viral interactions. Such a determination would probably best be made on a case-by-case basis.

4(b). **Please comment on the usefulness of the analyses described above for evaluating whether a PVCP-PIP meets (i) or (ii). Please describe any additional factors that the Agency could use in this evaluation (e.g., consideration of whether the plant virus species has an inherently low natural recombination frequency with respect to the coat protein gene).**

PVC-Protein Production Issues

When evaluating PVCP-PIPs for possible exemption under FIFRA, EPA must consider nontarget and human non-dietary risks from exposure to any potentially expressed PVC-proteins. Based on

the information currently available to the Agency to support an exemption, EPA is considering a criterion that describes PVC-proteins that are within the range of natural variation of the virus and therefore have a long history of safe nontarget and human exposure.

EPA consulted the 2004 SAP about possible nontarget effects of PVC-proteins. The panel confirmed that PVC-proteins within the range of natural variation of the virus would not be anticipated to present risks to nontarget organisms, concluding that, "[l]ethal effects in animal life after feeding on PVCP-PIP plants are highly unlikely because plant viruses are not known to have deleterious effects on animal life. Additionally, animals routinely feed on non-engineered virus-infected plants and do not die…. [S]ublethal effects are not expected to be manifested in animal life, again because wildlife and insects regularly feed on non-engineered virus-infected plants with no apparent sublethal damage" (U.S. Environmental Protection Agency 2004).

Based on the above advice, PVCP-PIPs that present low risk with respect to nontarget and human non-dietary exposures to PVC-proteins could be described by the following language: the genetic material encodes only a single contiguous portion of each unmodified viral coat protein. This would include multiple proteins expressed from a single PVCP-PIP construct, but not chimeric proteins. Under these conditions, such PVC-proteins would be identical to plant viral coat proteins that are widespread in plants and are not known to have any toxic effects on nontarget organisms or to have any toxic or allergenic effects in humans. Therefore, no nontarget or human non-dietary safety issues would be raised by PVC-proteins meeting this criterion.

The Agency recognizes that PVCP-PIP developers may wish to modify PVCP-PIP constructs to achieve certain product development goals such as greater efficacy, and such modifications might result in changes to the protein(s) produced. Many modifications to the genetic material may be so minor that they are unlikely to cause changes to the protein that would be significant from a human or nontarget organism perspective. Many of the modifications are likely to produce proteins that fall within the range of natural variation of the virus. However, it is not currently possible to *a priori* define a regulatory standard describing the range of variation of plant virus coat proteins in general or even of any particular virus. (See discussion in Unit II.B of Attachment II.) Given the large number and wide variety of changes that could be made to a genetic construct containing a plant viral coat protein gene and the differences among virus species in the amount of natural variation they exhibit, it would not be possible to decide *a priori* that any particular predetermined type or number of modifications would consistently produce a protein that falls within the range of natural variation. However, such a determination could be made on a case-by-case basis.

Therefore, PVCP-PIPs that present low risk with respect to nontarget and human non-dietary exposures to PVC-proteins could be described by the following language: the genetic material (i) encodes a protein that is minimally modified from a coat protein from a virus that naturally infects plants or (ii) produces no protein. In determining whether a PVC-protein is "minimally modified" from a natural viral coat protein, EPA would consider first whether the protein is substantially similar to a natural viral coat protein by evaluating information on the genetic construct, amino acid sequence, and molecular weight of the PVC-protein. EPA might also evaluate information developed by the submitter from public sequence databases on where the PVC-protein sequence falls relative to the range of natural variation. Those PVC-proteins that are determined to be substantially similar would be further evaluated to determine whether the

modified PVC-protein is as safe as an unmodified protein by considering information on the expression level of the PVC-protein relative to levels generally found in plants and information from amino acid sequence comparisons with known toxins and allergens. The type and extent of information that would need to be provided with an exemption request in order for EPA to determine whether a PVC-protein is "minimally modified" and therefore qualifies for the exemption would be determined on a case-by-case basis.

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

Post-Transcriptional Gene Silencing (PTGS) Issues

PVCP-PIPs may confer resistance in at least two different ways. In protein-mediated resistance, the coat protein is thought to impede the infection cycle by interfering with the disassembly of infecting viruses. In such cases, the PVC-protein appears to be the active ingredient directly effecting the pesticidal action. In other cases, prevention or mitigation of viral disease is not correlated with the level of coat protein expression, and RNA fragments appear to be the active ingredient, e.g., through post-transcriptional gene silencing (PTGS; Goldbach, 2003). Regardless of the mechanism of resistance, plants containing plant viral coat protein genes for the purpose of preventing or mitigating viral disease contain a pesticide subject to regulation by EPA under both FIFRA and FFDCA. EPA is seeking guidance from the SAP as to how the mechanism of resistance affects the evaluation of risk associated with PVCP-PIPs in order that the Agency can appropriately address all types of resistance mechanisms.

EPA is considering whether any proposed exemption under FIFRA should include criteria to address concerns associated with (1) gene flow, (2) viral interactions, and (3) exposure to PVC-proteins sufficiently different from those with which organisms generally interact when associating with the plant. Concerns associated with gene flow are not influenced by the mechanism of resistance based on the assumption that virus resistance could be expressed in a plant that acquired the genetic material regardless of whether resistance is protein- or RNA-mediated. However, concerns associated with recombination and PVC-proteins may be significantly reduced when resistance is RNA-mediated. For example, when a plant employs PTGS, RNA with sequence similarity to the transgene will be broken down, including that produced by the transgene (Goldbach et al. 2003). Thus, the potential for recombination with expressed transcripts from the transgene will be likewise reduced. The decrease in RNA also leads to a significant decrease in protein production (and therefore PVC-protein exposures). In some cases, protein production may be reduced to levels below the detection limit or even to zero.

Under FFDCA, any PVC-protein residues in food must be covered by a tolerance or tolerance exemption. Even when a PVCP-PIP prevents or mitigates viral disease through PTGS in which the pesticidal substance appears to be RNA, if any PVC-protein were to be produced in small

quantities, in certain tissues, at certain life stages, under certain environmental conditions, or in the case of suppression of gene silencing, this PVC-protein is part of the PVCP-PIP. (See Béclin et al. 1998; Mitter et al. 2001; Pang et al. 1996; Savenkov & Valkonen 2001; Szittya et al. 2003; and discussion in Unit II.D of Attachment II.)

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

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

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

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

Food Safety Issues

EPA's base of experience with viruses infecting food plants has led the Agency to draw three conclusions on which it would rely to support any tolerance exemption for residues of PVC-proteins in food. First, virus-infected plants have always been a part of the human and domestic animal food supply. Most crops are frequently infected with plant viruses, and food from these crops has been and is being consumed without adverse human or animal health effects. Second, plant viruses are not infectious to humans, including children and infants, or to other mammals. Third, plant virus coat proteins, while widespread in food, have not been associated with toxic effects to animals or humans. EPA believes these conclusions are derived from a sufficient

experience and information base to support a proposed exemption from the requirement of a food tolerance under FFDCA.

EPA is attempting to determine whether there would be any safety issues raised from exposure to PVC-proteins if the virus used to create the PVCP-PIP does not naturally infect the particular plant species into which the PVCP-PIP is inserted. A PVC-protein may be expressed in a food plant that the virus does not naturally infect when heterologous resistance to a particular virus is conferred through a different virus' coat protein gene (e.g., Dinant et al. 1993). Such situations may also arise when a small segment of a plant virus coat protein gene is used to achieve expression of a coat protein gene from a different virus (e.g., Gonsalves 1998). The Agency is attempting to determine whether such PVC-proteins present low dietary risk based on the rationale that these proteins are reasonably expected to be part of the current diet. Based on their broad host range, plant viruses are known generally to infect a wide variety of plants that humans consume. People generally eat a broad range of food plants through which they would reasonably be expected to be exposed to a wide variety of plant virus coat proteins. In addition, EPA is not aware that any plant viral coat proteins have been identified as allergens, so it is unlikely that a person with food allergies avoids a particular food plant because of an allergic reaction to a viral coat protein. Based on this rationale and in the absence of contravening evidence, EPA believes that a PVC-protein expressed in a plant that is not normally infected by the corresponding virus would raise no safety issues.

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

The rationale for exempting truncated PVC-proteins from food tolerance requirements would be that segments of coat proteins exist in nature due to processes such as incomplete translation of transcripts and partial degradation of proteins. Incomplete translation may occur due to routine replication errors causing a ribosome to dissociate from an RNA transcript or if mutation introduces a premature stop codon, i.e., a nonsense mutation. Truncated plant virus coat proteins are indeed known to occur in nature (Sacher & Ahlquist 1989). Thus, PVC-proteins that are truncated forms of naturally occurring plant virus coat proteins would not significantly increase the likelihood of exposure to a toxic or allergenic protein since humans are currently exposed to them in the diet along with complete plant virus coat proteins.

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

The AUG codon for methionine initiates translation in eukaryotes (Berg et al. 2002). Among certain viruses such as the Potyviridae, the coat protein is produced as part of a polyprotein, so the coding region for the coat protein is excised from the genetic material encoding the polyprotein to create a PVCP-PIP and thus normally lacks a start codon. Insertion of an AUG codon allows for PVC-protein expression, which may be needed to confer virus resistance. EPA believes the addition of a single, N-terminal methionine residue would be unlikely to affect a PVC-protein's toxicity or allergenicity relative to a naturally occurring plant virus coat protein.

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

Viruses have a wide range of natural variation and it is likely that many modifications in addition to truncations and the addition of an AUG codon could be introduced into the genetic material encoding the PVC-protein and result in exposure to PVC-proteins similar to plant viral coat proteins currently in the diet. However, protein modifications have been recognized as having the potential to significantly alter a protein's properties.

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

Under EPA's current approach, in determining whether a PVC-protein is "minimally modified" from a natural viral coat protein, the Agency would consider first whether the protein is substantially similar to a natural viral coat protein by evaluating information on the genetic construct, amino acid sequence, and molecular weight of the PVC-protein. EPA might also evaluate information developed by the submitter from public sequence databases on where the PVC-protein sequence falls relative to the range of natural variation. Those PVC-proteins that are determined to be substantially similar would be further evaluated to determine whether the modified PVC-protein is as safe as an unmodified protein by considering information on the expression level of the PVC-protein relative to levels generally found in plants humans consume and information from an amino acid sequence comparison with known toxins and allergens. The type and extent of information that would need to be provided in order for EPA to determine whether a PVC-protein is "minimally modified" would be determined on a case-by-case basis.

8. **Please comment on the usefulness of the factors described above for evaluating food safety of the encoded PVC-protein. How important is it to characterize the expressed**

protein, e.g., to determine whether any post-translational modifications have occurred?

Some PVC-proteins may be chimeric proteins that are encoded by a sequence constructed from portions of two or more different plant virus coat protein genes. Such constructs may be made to enable appropriate expression of the desired coat protein gene and confer resistance in the plant (Gonsalves 1998; Ravelonandro et al. 1992) or to expand the range of viruses to which the plant is resistant (Lindbo et al. 1993).

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

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

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

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

Other Issues

Under the regulatory structure established for PIPs, selectable markers (i.e., inert ingredients by definition under FIFRA) are considered to be part of the PIP. EPA has identified three selectable markers that it believes present a low probability of risk to human health and the environment when used in any plant on the list in question 1(a): CP4 enolpyruvylshikimate-3-phosphate (CP4 EPSPS), glyphosate oxidoreductase (GOX or GOXv247), and phosphinothricin acetyltransferase (PAT). In addition, EPA has identified three selectable markers that it believes pose low risk to human health and the environment when used in any plant as part of a PIP: beta-D-glucuronidase (from *E. coli*), neomycin phosphotransferase II (NPTII), and phosphomannose isomerase (PMI). Each of these proteins already has a tolerance exemption under FFDCA section 408. The Agency is now considering exempting these selectable markers from regulation under FIFRA.

10. **Please comment on the Agency's environmental risk assessment of each of the six selectable markers (found in attachment III). Does the SAP concur that CP4 EPSPS, GOX/GOXv247, PAT each pose a low probability of risk to the environment when**

used in one of the plants listed in question 1(a)? Does the SAP concur that beta-D-glucuronidase, NPTII, and PMI each pose a low probability of risk to the environment when used in any plant?

In examining approaches to identifying and describing PVCP-PIPs likely to present low risk, EPA has developed criteria to address the risk issues most commonly identified as potentially associated with PVCP-PIPs. The SAP has been asked to comment on aspects of these criteria in this meeting. The Agency recognizes that other viral components have been or are being used to develop PIPs intended for use in protecting against viral disease, e.g., PIPs based on viral replicase genes.

The Agency asked the SAP in 1992 to what extent the rationale used in the proposed exemption for PVCP-PIPs could be applied to other viral components. The SAP responded:

> "Other viral gene products usually are expressed at lower levels in the infected plants than viral coat protein. Also, their turnover rate is much higher. Thus, in some cases, transgenic plants expressing other viral-encoded proteins may express these proteins at levels higher than in a naturally infected plant. Hence, a generalization cannot be made for such proteins as can be made for coat protein. Although at present this approach seems reasonable, additional research will be necessary before any generalizations can be made" (U.S. Environmental Protection Agency 1992).

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

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