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

I. What Action Does this Paper Discuss?

EPA is considering whether to establish a tolerance exemption under section 408 of the Federal Food, Drug, and Cosmetic Act (FFDCA) for residues of coat proteins from viruses that naturally infect plants that humans consume, when such coat proteins are produced in living plants as part of a plant-incorporated protectant and when certain criteria are met. The criteria EPA is considering are intended to clearly identify and exempt only those residues for which a long history of safe exposure and consumption can support exemption. EPA is still considering several scientific issues that would affect the exact formulation of these criteria, and these issues are discussed below in this document. EPA believes there is a reasonable certainty that no harm will result from aggregate exposure to such residues, including all anticipated dietary exposures and all other exposures for which there is reliable information. A tolerance exemption would eliminate the need to establish a maximum permissible level in food for these residues.

II. Background

Coat proteins are those substances that viruses produce to encapsulate and protect the viral nucleic acid and to perform other important tasks for the virus, e.g., assistance in viral replication, movement within the plant, and transmission of the virus from plant to plant by insects (Ref. 1). Current scientific information suggests that prevention or mitigation of disease by some PVCP-PIPs may be protein-mediated because for certain PVCP-PIPs efficacy is correlated with the concentration of coat protein produced by the transgene (Ref. 2). In protein-mediated resistance, the coat protein portion of the PVCP-PIP (hereafter the "PVC-protein") is thought to impede the infection cycle by interfering with the disassembly of infecting viruses (Ref. 3). In such cases, the PVC-protein appears to be the active ingredient directly effecting the pesticidal action. Residues of such PVC-proteins and their metabolites and degradates would be the subject of this action if they met the specified criteria.

In transgenic plants where post-transcriptional gene silencing (PTGS) has been activated, prevention or mitigation of viral disease is not correlated with the level of PVC-protein expression. Indeed, virus resistance can occur even when a coat protein gene expresses untranslatable RNA sequences and no PVC-protein is detected. In PTGS, RNA fragments appear to be the active ingredient directly effecting the pesticidal action (Ref. 3). Even when PTGS is the mechanism of resistance, should any PVC-protein be produced, this PVC-protein is part of the PVCP-PIP. Residues of such PVC-proteins and their metabolites and degradates would also be the subject of this action if they met the specified criteria.

A. Potential Rationale Supporting an FFDCA Tolerance Exemption

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.

1. *Always been part of food supply without adverse effects*

Virus-infected food plants have always been a part of the human and domestic animal food supply (Refs. 4, 5, 6, 7, 8, 9). Most plants are infected by at least one virus, and components of plant viruses, including coat proteins, are often found in the produce of crop plants. For example, at the beginning of this century virtually every commercial cultivar of potatoes grown in the United States and Europe was infected with either one or a complex of potato viruses (Ref. 9). Even plants that show no disease symptoms are often found to be infected with viruses (Refs. 8, 10). In addition, a common agricultural practice used since the 1920s for protection against viruses involves intentionally inoculating healthy plants with a mild form of a virus in order to prevent infection by a more virulent form (Ref. 10). A great deal of information supports the ubiquitous appearance of plant viruses in foods, and to date there have been no reports of adverse human or animal health effects associated with consumption of plant viruses in food.

The National Research Council (NRC) observed in its 2000 report that "[h]uman or animal consumption of plants with viral coat proteins is widely considered to be safe, on the basis of common exposure to these types of proteins in nontransgenic types of food" (Ref. 11). The FIFRA SAP at its December 18, 1992 meeting (Ref. 12), also addressed the issue of dietary risk. The SAP stated that "[s]ince viruses are ubiquitous in the agricultural environment at levels higher than will be present in transgenic plants, and there has been a long history of 'contamination' of the food supply by virus coat protein, there is scientific rationale for exempting transgenic plants expressing virus coat protein from the requirement of a tolerance." The FIFRA SAP again discussed PVC-proteins on October 11-13, 2004 and "agreed that (because of the human history of consuming virus infected food), unaltered PVCPs do not present new dietary exposures" (Ref. 13).

In general, EPA anticipates that dietary exposure through human and animal consumption of plants containing residues of PVC-proteins that EPA is considering exempting will be similar to or less than the dietary exposure to plant virus coat proteins currently found in food plants naturally infected with viruses. Experiments have shown that PVC-protein levels in plants resistant to a virus because of a PVCP-PIP, even when the resistance is mediated by the PVC-protein itself, can be up to one hundred- to one thousand-fold lower in concentration than the level of coat protein found in plants naturally infected by viruses (Refs. 7, 14). The difference in amount of PVC-protein present is even more marked for virus-resistant plants employing resistance mediated by RNA. In such cases, little to no detectable coat protein is produced in a plant containing a PVCP-

PIP (Refs. 3, 15). Such information conforms to information EPA has received from the scientific advisory groups the Agency has consulted.

2. *Not infectious to humans*

Any virus/host relationship is characterized by a high degree of specificity (Ref. 7). Plant viruses usually infect plants only within a certain taxonomic group and are unable to infect humans or other vertebrates (Refs. 16, 17). Cellular machinery for processing genetic material is highly specific. For example, plant viruses are unable to recognize and attach to the specific sites on mammalian cells needed to penetrate the cell membrane, and plant viruses cannot be processed by mammalian cellular machinery. Plant viruses therefore do not and cannot infect mammals and other vertebrates. In addition, multiple virus components in addition to the coat protein have a role in and are necessary for plant infection. Plant viral coat proteins alone are not infectious to plants, and whole, intact plant viruses are not infectious to humans. Therefore, it is reasonable to assume that a single component of plant viruses, e.g., the PVC-protein, will not be infectious to humans.

3. *No toxic effects to animals or humans*

Humans and domestic animals have been and are exposed to plant viruses in the food supply because most crops are frequently infected with plant viruses. Food from these crops has been and is being consumed without human or animal toxicity related to plant virus infections. Additional evidence of a lack of toxicity can be deduced from the common practice of injecting laboratory animals with purified plant virus preparations without any adverse effects on the animals (Ref. 15). Furthermore, the Agency is not aware of any coat protein from a virus that naturally infects plants that has been identified as a food allergen for humans. Finally, the amount of PVC-protein likely to be found in food is anticipated to be lower than the amount of virus coat protein found in food naturally infected with plant viruses (as discussed in Unit II.A.1).

B. Key Issue: Determination of Natural Virus Variation

The key issue facing EPA in developing an exemption is how to describe clearly for regulatory purposes those PVC-proteins that are within the range of naturally occurring plant virus coat proteins and to which the rationale discussed in Unit II.A therefore applies. If a plant virus coat protein gene is isolated in nature and not modified, the PVC-protein would clearly be within the range of natural variation. However, many coat protein genes are modified in creating a PVCP-PIP, e.g., to increase product efficacy or allow appropriate expression in the plant. Some of these modifications may affect a PVC-protein, although most of these variations would not be expected to differ significantly (e.g., in terms of toxicity or allergenicity) from the naturally occurring coat protein. In fact, given the considerable variation in naturally occurring viral coat proteins, it is also

possible that naturally occurring plant viruses exist with some of the minor modifications that could reasonably be anticipated.

EPA's task of defining this variation is complicated by the variable nature of plant virus populations and the fact that the full extent of variation for even a single plant virus is currently unknown. Sequencing of plant virus genomes has revealed that a large number of variants exist within most populations of both RNA and DNA viruses. Due to this inherent heterogeneity in virus populations, they are often described as "quasispecies" that exist as a pool of different sequences varying around a consensus sequence (Refs. 18, 19, 20).

Genetic variation in virus populations arises due to several processes including mutation, recombination, and reassortment. Mutation is a change in the genetic material that most commonly occurs when replication errors lead to incorporation of an incorrect nucleotide into the daughter sequence (Ref. 21). New virus variants are also generated by recombination, the natural process that occurs during replication of DNA or RNA whereby new combinations of genes are produced. Recombination is more likely to occur the more closely related viruses are. However, recombination between different viral species is also believed to occur and to have generated new viruses (Refs. 22, 23). Evidence of past recombination having led to the creation of new DNA and RNA viruses has been found in a number of different groups including bromoviruses (Ref. 24), caulimoviruses (Ref. 25), luteoviruses (Ref. 26), nepoviruses (Ref. 27), cucumoviruses (Ref. 28), and geminiviruses (22, Refs. 29). Sequence analysis of viruses from the family Luteoviridae indicated that this family has evolved via both intra- and interfamilial recombination (Ref. 30). In viruses with segmented genomes, variation may also be caused by reassortment whereby entire segments are exchanged between viruses (Ref. 31).

Attempts to describe the range of variation for naturally occurring plant virus coat proteins are complicated not only by variation within species but also by variation among species (See ref. 32 for review). For example, cucumber mosaic cucumovirus (CMV) has a relatively high degree of variation (Ref. 33) compared to tobacco mild green mosaic tobomovirus (Ref. 34). The greater variability in CMV would be expected based on the relatively wide host range and relatively high recombination rate of this virus.

A large number of viral coat protein sequences are currently available in the literature and in public sequence repositories, e.g., the National Center for Biotechnology Information. However, EPA has concluded that it is not possible to use this information to establish a regulatory standard. One possibility that EPA considered but ultimately rejected was to determine an idealized sequence for each virus species in which each position of the sequence represents the amino acid most often found when all available sequences are compared. A selection procedure would be used to determine which amino acid is placed at a given position in the event that not all of the sequences have the identical amino acid at that position. The percentage of positions that each sequence deviates from this idealized sequence could be calculated to establish a maximum deviation found in nature. A percent deviation could likewise be calculated for a PVC-protein sequence to determine if it differed by more or less than the maximum found in nature. One problem with such an approach is that focusing only on a percent deviation does not take into account important information about which regions of the coat protein vary and which regions are highly conserved. For example, a particular sequence constructed in a laboratory by modifying a

natural variant could differ from the idealized sequence by less than the prior determined percent variability for natural variants, but the particular sequence change could still be outside the range of natural variation depending on the region that was modified. In addition, the maximum variation is likely to change over time as additional sequences are determined and as viruses evolve. EPA does not believe it could develop a standard that would take this sort of information into account. Moreover, no single standard could capture the degree of variation across all viruses, and hundreds of plant viruses have been identified to date (Ref. 35). It would be at best impractical for EPA to describe individually for all virus groups all potential modifications that would produce a PVC-protein that falls within the range of natural variation given the vast (and yet still incomplete) amount of data that currently exists.

Consequently, at the present time, insufficient information exists to develop a standard that would describe *a priori* the degree to which a PVC-protein could be modified and yet still remain within the natural variability of plant virus coat proteins found in virus populations either generally or for any species in particular. In light of this, EPA is considering an approach to exempt PVC-protein residues from the requirement of a tolerance by: (1) a categorical exemption for a subset of PVC-proteins based on developer self-determination that the encoded PVC-protein is identical to any single contiguous portion of an unmodified coat protein from a virus that naturally infects plants that humans consume in toto or in part, and (2) an exemption for more extensively modified proteins that is conditional on an Agency determination after review that the encoded PVC-protein is minimally modified from an unmodified coat protein from a virus that naturally infects plants that humans consume in toto or in part.

C. Potential Exemption Structure

1. Categorical exemption

When the encoded PVC-protein is identical to any single contiguous portion of an unmodified coat protein from a virus that naturally infects plants that humans consume in toto or in part, the developer may determine that the residues of the PVC-protein would be exempt from the requirement of a tolerance without Agency review. If the PVC-protein is expressed from a plant virus coat protein gene that was isolated in nature from a food plant and was not modified, the PVC-protein would meet this criterion. Additionally, if the genetic material encoding the PVC-protein has been modified but its amino acid sequence nevertheless exactly matches a database sequence from an unmodified plant virus coat protein that naturally infects plants that humans consume, the PVC-protein would meet this criterion. Although EPA cannot *a priori* identify all existing natural coat protein variants, the requirement of identity with any single contiguous portion of an unmodified coat protein would ensure that the exempted PVC-protein falls within the existing base of experience on which any exemption would rely.

Under this approach, EPA intends to exclude from the categorical exemption residues of modified PVC-proteins, e.g., PVC-proteins containing insertions, internal deletions, or amino acid substitutions, as well as chimeric PVC-proteins that are encoded by a sequence constructed by fusing portions of two or more plant virus coat protein genes. EPA is considering excluding such PVC-proteins from the categorical exemption because insufficient information exists at this time

to allow EPA to describe *a priori* a single standard articulating which of these types of changes would be consistently expected to fall within the natural range of variation of viruses and/or which types of changes could be determined not to affect toxicity or allergenicity without any EPA review of the protein and/or construct.

Segments of PVC-proteins that are identical to segments of an unmodified coat protein would be exempted categorically under this approach, i.e., without Agency review. EPA believes the exemption of segments would be supported by the experience base EPA discussed above to support an exemption. It is probable 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 (Ref. 36). 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.

The Agency is considering whether also to include in the categorical exemption, i.e., without Agency review, amino acid sequences containing terminal deletion(s) and/or an additional N-terminal methionine residue. The AUG codon for methionine initiates translation in eukaryotes (Ref. 37). 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.

Under this approach EPA would require that the virus used as the source of the coat protein sequence naturally infects plants that humans consume as an additional means of ensuring that any exemption is limited to PVCP-PIPs that fall within the base of experience discussed previously in this paper. EPA would limit the proposed exemption to residues of PVC-proteins that are already part of the human diet as naturally occurring plant virus coat proteins or because they are minimally modified from such proteins. For example, the exemption would not extend to PVC-proteins encoded in part by sequences from animal or human viruses. EPA means by the phrase "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."

EPA is considering whether to limit any exemption to PVC-proteins from PVCP-PIPs based on viruses that naturally infect the *particular* food plant in which the PVC-protein is expressed. EPA must address 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., Ref. 38). 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., Ref. 39). However, such PVC-proteins could be safely exempted from tolerance requirements because these proteins could be 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. Therefore, a PVC-protein expressed in a plant that is not normally infected by the corresponding virus would raise no safety issues as long as the corresponding virus infects plants consumed by humans.

EPA is also considering whether a geographic limitation on any categorical exemption would also be necessary to ensure that the exemption extends only to residues that are part of the U.S. diet; i.e., that any exemption would only extend to PVC-proteins that are part of a PVCP-PIP constructed from a virus that occurs naturally in the United States. Such a limitation would not be necessary to ensure that any exempted PVC-proteins fall within the base of experience supporting the exemption. Humans have long consumed viruses infecting food plants with no adverse effects. Given modern market practices in which food is shipped globally for consumption by people of diverse nationalities, broad, transnational human dietary exposure to all viruses that infect plants humans consume is likely and has been associated with no known adverse effects.

2. Exemption conditional on Agency determination

Product developers frequently modify the genetic material of a PVCP-PIP, e.g., in order to achieve greater efficacy (Ref. 40). Some of these modifications may affect the PVC-protein, although many of these protein changes may be so minor that they are unlikely to significantly affect potential dietary risk. However, at this time the Agency cannot articulate a criterion that would ensure all PVC-proteins with such modifications fall within the base of experience discussed above in Unit II.A.

The question of how to objectively define criteria on which the regulated community may rely to determine *a priori* how much a virus coat protein may be modified and still fall within the range of natural variation is a key challenge. EPA first considered the question of how to describe residues that fall within the base of experience supporting exemption when the Agency issued its proposal on November 23, 1994 (59 FR at 60539). In the July 19, 2001 supplemental notice (66 FR

37865), EPA again addressed the question of how to describe PVCP-PIPs that fall within the recognized base of experience supporting an exemption.

In October 2004, the FIFRA SAP was asked to consider the degree and ways a PVCP gene might be modified while still retaining scientific support for the idea that humans have consumed the products of such genes for generations and that such products therefore present no new dietary exposures (Ref. 13). They responded that "[t]here was no clear consensus on how much change would be necessary to invalidate this assumption, although there was general agreement that the appropriate comparison is to the range of natural variation in the virus population."

Developing objectively defined criteria on which the regulated community could rely to determine whether a modified PVC-protein falls within the natural range of variation for a particular virus may not be currently feasible because the Agency knows of no generally applicable, established baseline for what constitutes the range of natural variation of a virus. EPA thus does not believe that an exemption that would allow developers to self-determine eligibility of modified PVC-proteins would be supportable. Rather, EPA is considering an option under which the residues of such a PVC-protein would be exempt only if the Agency determines after review that the encoded PVC-protein is minimally modified from an unmodified coat protein from a virus that naturally infects plants that humans consume in toto or in part.

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 humans consume and information from amino acid sequence comparisons 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.

For residues of PVC-proteins that would not qualify for either the categorical or conditional exemption discussed above, an applicant would be able to petition the Agency for an individual tolerance exemption under FFDCA section 408 (See also 40 CFR 180.7).

D. Tolerance Issues Associated with Post-Transcriptional Gene Silencing

Questions remain about circumstances under which PVC-protein might be detected and/or produced in food at some point after commercialization even though PVC-protein may not have been detected and/or produced during product development. For example, it is known that in some cases PTGS must be triggered before transgene RNA production can be effectively suppressed. Lindbo *et al.* (Ref. 41) used tobacco etch virus (TEV) to infect transgenic tobacco plants containing a TEV coat protein gene. Plants temporarily developed symptoms but were able

to recover from infection. Recovered transgenic plant tissue showed significantly reduced levels of transgene mRNA, and PVC-protein was undetectable. However, plant tissues unchallenged with virus did express PVC-protein, suggesting that in at least some cases of PTGS-induced virus resistance, PVC-protein may be produced until virus infection occurs. Béclin *et al.* (Ref. 42) showed that in transgenic tobacco lines expressing a β-glucuronidase (uidA) transgene, suppression of transgene expression always occurs but is initiated at different plant developmental stages: either 15 days after germination or two months post-germination. Prior to PTGS initiation, transgenic protein is expressed, suggesting that in at least some cases lack of protein production may only occur after a certain developmental stage is reached. Likewise, Pang et al. (Ref. 43) found that plant developmental stage plays an important role in the timing of PTGS initiation.

Experiments demonstrating that plant developmental stage determines PTGS initiation suggest that any environmental factors influencing plant growth would also affect the amount of time before RNA and possibly protein production is effectively suppressed. At least one experiment has looked more directly at the influence of environmental factors on PTGS. Szittya et al. (Ref. 44) demonstrated that cold temperatures inhibited transgene-induced RNA silencing leading to increased levels of transgene mRNA, although the level of transgenic protein was not reported.

In addition to temporal changes in protein production that may be influenced by varying environmental conditions, PTGS may also be associated with variation in protein expression across different plant tissues. Plant lines expressing a nitrate reductase transgene were found to display PTGS in leaves and stem tissue but not in shoot apical or axillary meristems (Ref. 42). As in other experiments (Ref. 41), transgene protein was not detectable and transgene mRNA levels were significantly reduced in plant tissue displaying PTGS. However, plant tissue in which gene silencing does not occur showed normal levels of transgene mRNA, and transgenic protein was produced.

It is known that PTGS can be suppressed leading to loss of the virus-resistant phenotype conferred by a PVCP-PIP. For example, Savenkov and Valkonen (Ref. 45) showed that resistance to Potato virus A (PVA) in *Nicotiana benthiana* could be overcome when plants were challenged with Potato virus Y (PVY). Although levels of transgene mRNA in healthy transgenic plants was extremely low or below the detection limit, it was readily detectable in PVY-infected plants where suppression of gene silencing had apparently occurred. The study did not report whether PVC-protein was produced from the transgene mRNA.

Such experiments suggest that many factors should be considered in making a determination of whether a PVC-protein might be produced after commercialization. Some characteristics of the PVCP-PIP, e.g., one that produces untranslatable or antisense coat protein transcripts, may offer a reasonable level of assurance that PVC-protein production would not occur.

III. References

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