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Comments by Seminis Vegetable Seeds
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To Whom It May Concern:

Seminis Vegetable Seeds, Inc. commercialized the first genetically engineered (biotech) crop (ZW-20) containing viral coat protein genes in 1995, after receiving the following decisions from government agencies:

- USDA Determination of Non-Regulated Status 12/07/94
- FDA Completion of Consultation 4/5/95
- EPA Exemption from the requirement of a tolerance 11/2/94
- Canadian Food Inspection Agency for fruit importation 12/2/97
- Health Canada approval 4/17/98

ZW-20 squash contains coat protein genes from Zucchini Yellows Mosaic Virus and Watermelon Mosaic Virus 2.

Subsequently, Seminis commercialized a second genetically engineered squash line (CZW-3) containing three viral coat protein genes in 2000, after receiving the following decisions from government agencies:

- USDA Determination of Non-Regulated Status 5/6/97
- FDA Completion of Consultation 7/11/97
- EPA Exemption from the requirement of a tolerance 8/14/97
- Canadian Food Inspection Agency for fruit importation 12/2/97
- Health Canada approval 4/17/98

CZW-3 squash contains coat protein genes from Cucumber Mosaic Virus, Zucchini Yellows Mosaic Virus and Watermelon Mosaic Virus 2.

These products have been well received by squash farmers and provide excellent resistance to the indicated plant viruses. Natural resistance to these viruses is significantly inferior and does not provide the same high level of virus resistance. In many cases, farmers have reported success in producing a squash crop using the biotech varieties whereas they had crop failure using other varieties due to these viruses.

USDA APHIS oversight is sufficient and adequate

USDA APHIS conducted a rigorous 2-year review prior to its Determination of Non-Regulated Status for the virus-resistant ZW-20 squash line (and later, a similar review for CZW-3). The Determination was supported by several years of data accumulation from initial contained tests in the greenhouse, and small-scale field trials to large-scale field trials. These tests included studies on gene flow and viral interactions such as encapsidation. During the deregulation process USDA solicited public comments on the petition and the comments received were carefully considered. Furthermore, USDA consulted state extension agents in relevant states and USDA requested additional studies from Asgrow (now part of Seminis Vegetable Seeds), which were performed. All this was done in addition to the usual independent scientific literature survey and expert consultations USDA routinely does on top of the voluminous information requested of petitioners.

The issues before this EPA Science Advisory Panel are important questions, which USDA APHIS has considered when making its Determinations of Non-Regulated Status for PVCP-PIPs, specifically in the areas of gene flow and viral interactions. USDA APHIS has now a 12-year history of making PVCP-PIP Determinations. Under FFDCA and FIFRA, EPA can finalize an exemption from regulation for PVCP-PIPs and not create an unnecessary and duplicative registration process that USDA APHIS adequately covers.

Loss of product

Since 1994, Seminis has conducted research on other PVCP-PIP vegetables, including tomato and melon. In most cases, the PVCP-PIPs resulted in biotech plants that had excellent resistance to the target viruses. If such resistance had come from wild relatives via wide hybridization techniques, as has been done in plant breeding for decades, these virus resistant varieties would have been commercialized and farmers today would be benefiting from improved virus resistance. However, because of the regulatory costs to obtain approvals of PVCP-PIPs, Seminis determined that these other PVCP-PIPs would not be able to justify sufficient value-added to pay for the regulatory approvals. Therefore, the vegetable PVCP-PIP projects were discontinued, which is unfortunate for farmers who still must battle virus infestations.

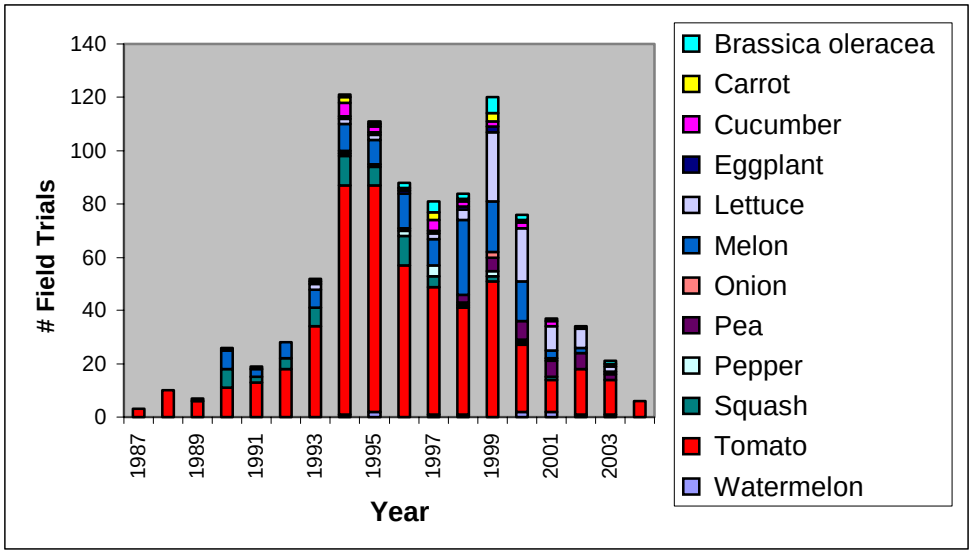
It should be noted, that under today's regulatory burden, Seminis would not be able to justify the investment to create our two biotech squash products, ZW-20 and CZW-3, which are currently providing significant benefit to American farmers. Squash is not a high-value crop. The premium that farmers are willing to pay for virus resistant squash is simply not enough to cover today's regulatory expenses. In fact, because of PIP registration costs, it is likely that these virus resistant squash varieties would have to be removed from the market should they no longer be exempt from the requirement of a tolerance. This would place a significant burden on those farmers who have come to depend on these varieties in order to produce marketable crops.

Regulatory burden

Development of biotech horticultural crops (minor crops) has slowed significantly over the past five years, as seen by a decrease in field trials of horticultural crops (see figure below) and a cessation or severe reduction in research activity and product development in industry. Even academic scientists are wondering if it will ever be possible for them to release the biotech varieties they are developing in the horticultural field. There are

several reasons for this, including the European Union's moratorium on biotech approvals, lack of tolerance levels for adventitious presence in seed, and, of importance to this Science Advisory Panel and the EPA, significantly increased regulatory costs. While progress in the US has slowed and all approvals in the EU have stopped until just recently, some countries, such as China, continue to develop biotech products for their internal markets. It is predicted that within a few years, China will emerge as the leader in biotech horticultural crops.

US field trials from 1987 to February 2004 (Redenbaugh, K. and A. McHughen. 2004. Regulatory challenges reduce opportunities for horticultural biotechnology. California Agriculture 58:106-115), compiled from http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm



The regulatory requirements to develop improved biotech varieties (over-and-above the costs to develop traditionally bred varieties) is now at least $1 million per allele (if limited strictly to the US) and more likely at $5 million or more per allele, depending on the number of countries in which approvals are required (an allele or genetic trait is a transformation event). Due to the international trade in horticultural commodities, there are few examples of products under development in which both the seed and the product could be contained solely in the US. More likely, a biotech variety will need approvals in a number of countries to which the product might be exported. For example, biotech processing tomatoes grown in California would end up being exported as tomato paste or other products to many countries around the world, each of which must give food approval prior to commercialization. And, if the processed product contains seeds that might be viable, environmental studies and approvals will also be required in the importing country, even if the importation is intended only for food consumption. Importing countries may also impose additional and unique requirements, such as labeling or the ability to trace the product back to the producing farm, as in the EU.

Any additional regulatory burden, such as registration of PVCP-PIPs, would further reduce the opportunities to develop biotech horticultural crops.

Seminis request and recommendation

Seminis strongly recommends that the EPA continue to provide exemption from regulation for PVCP-PIPs. USDA adequately addresses the issues raised by EPA, including gene flow, transencapsidation and recombination and has held numerous public fora and workshops to gather information and obtain public input on PVCP-PIPs. EPA registration is unnecessary and duplicative of the USDA oversight it has provided for 12 years.

Seminis responses to FIFRA SAP questions

1. What scientific evidence supports or refutes the idea that plant viruses have significant effects on reproduction, survival, and growth of plant populations in natural settings? Is there scientific evidence that plant populations freed from viral pressure could have increased competitive ability leading to changes in plant population dynamics?

   Research by Fuchs and Gonsalves with commercial biotech squash provided evidence that viruses are not limiting the growth, and fruit and seed production of a wild population of squash. They concluded that "there is little, if any, evidence that the introduction of virus resistance could provide wild squash species with a tremendous selective advantage." See attached report from the 8th International Symposium on the Biosafety of Genetically Modified Organisms.

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

   Seminis has extensive experience with tomato and sweet corn, neither of which have wild or weedy relatives in the United States.

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

   There are no wild or weedy relatives of beans (*Phaseolus vulgaris*), cucumber, eggplant, onion, pea, pepper, and spinach in the United States. *Brassica oleracea* (cauliflower, broccoli, cabbage, etc.) has wild relatives, but embryo rescue is usually required for such crosses.

   There are no cucurbit crops (squash, melon, pumpkin) with conspecific weedy relatives in the United States. All of the conspecific wild cucurbits are insignificant with respect to weediness.

4. What laboratory techniques used to achieve genetic exchange between species (e.g., embryo rescue, use of intermediate bridging crosses, protoplast fusion) are not indicative of possible genetic exchange between these species in the field? Conversely, what techniques, if any, used in laboratory or greenhouse experiments provide the most reliable indication of ability to hybridize in the field?

   Species, which can only be crossed using embryo rescue, use of intermediate bridging crosses, hand crossing, protoplast fusion, etc. are of little concern for gene flow in natural settings. Such species should not be considered as potential recipients of transgenes in the natural environment. For laboratory or greenhouse experiments, appropriate techniques for ability to hybridize are wind and insect pollination. Wind pollination can be simulated by providing air flow within a greenhouse or via mechanical means. For insect pollination, the appropriate pollinator for the species being studied must be chosen.

   It is possible to use embryo rescue and/or bridging crosses to transfer genes between several members of the genus *Cucurbita*, but the intermediate

individuals are sterile and/or remarkably weak, such that they would not survive in a natural environment.

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

   The movement of genes between sexually compatible species has occurred for centuries using common breeding practices. A major breeding strategy in traditional plant improvement programs is to significantly increase the level of natural PIPs to combat disease, insects or other pests. Seminis is unaware of any traditional breeding efforts for virus resistance that have led to the transfer of the PIP with resultant negative impact on the environment.

   Since EPA has determined that PIPs in traditionally bred plants are exempt from all FIFRA requirements (except for an adverse effects reporting requirement), it follows that these same PIPs moved to other sexually compatible plants, regardless of method, should also be exempt. Furthermore, should there be an adverse effect, the developer would be required by FIFRA to report the effect, at which time the Agency would respond accordingly. The potential of increasing a PIP is equally great in sexually compatible species, regardless of the method for transferring the PIP.

   See also response to Question 6.

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

   The majority of traditional viral resistance (if not all) in commercial vegetable crops such as tomato, pepper and squash originated in wild species. Examples include resistance to tobamovirus, geminivirus, potyvirus and tospovirus in tomato and pepper and resistance to CMV, potyvirus and geminivirus in squash. Wild relatives of *Cucurbita* spp. crops include buffalo gourd and swamp gourd. The swamp gourd (*C. okeechobeensis*) is the original source for resistance to some viruses that is used by traditional breeding techniques.

   Biotech virus-resistant squash has been commercially planted for 9 years in the United States. During this time, there have been no reports of squash or wild relatives becoming a pest problem. There are no reports of wild or weedy squash relatives gaining a selective advantage due to proximity to virus-resistant squash fields. Farmers are often quick to report back to seed companies on any problems, such as those just described, but Seminis has received no farmer complaints on these issues. The observations over the past 9 years confirm the validity and completeness of the USDA Determinations of Non-Regulated Status as well as supporting EPA's 1994 proposed rule exempting from tolerance viral coat protein genes.

   The USDA acknowledged that pollen flow will likely occur between biotech and wild squash and that movement of virus resistance genes into the wild populations were likely to be retained in a population if the genes confer an advantage to the plant containing the new genes. The evidence from Seminis'

virus survey of wild populations indicated that there is not continuous virus pressure on these wild populations and under those circumstances, wild population with the virus resistance gene will not have any selective advantage over wild populations without the virus genes. Therefore the selective pressure to maintain the virus-resistant trait in the wild population will be minimal.

Natural populations of free living *Cucurbita* populations (FLCP) appear to be largely free of infection by CMV, ZYMV and WMV2, strongly suggesting that resistance to CMV, ZYMV and WMV2 would not provide any selective advantage. Should the virus resistance genes from Seminis biotech squash transfer to FLCP, the selective pressure to maintain the virus resistance in natural populations of FLCP would be minimal, since all evidence supports the conclusion that FLCP are not under significant environmental stress from viral infection.

While these types of studies are useful for determining the environmental safety of each new virus-resistant, biotech crop, the oversight provided by the USDA ensures that all these issues are considered and evaluated.

EPA's suggestion that the Agency could require PVCP-PIP registration would directly duplicate what USDA requires. Therefore, a duplicate submission to EPA would not be necessary. It is Seminis' opinion that, based on USDA regulatory oversight, all PVCP-PIPs should be exempted from tolerance.

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

   Mechanical inoculation and subsequent incubation in greenhouse conditions, in general, provide measures of resistance that are relevant to field conditions.

   Visual ratings of symptoms are the preferred method in squash for evaluating resistance to ZYMV, WMV, CMV, PRSV (Papaya Ringspot Virus), SqLCV (Squash Leaf Curl Virus), and other viruses in the field. Symptoms of infection are generally severe and obvious, resulting in plant distortion, discoloration, and possibly death. ELISA techniques can be used to identify which viruses are present, but this technique does not necessarily confirm which virus is causing the symptoms observed. Plants which are resistant to symptom development may serve as a host for a particular virus, without displaying severe symptoms, so ELISA alone cannot be used to determine resistance.

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

   Environmental factors strongly affect epiphytotics of disease, primarily by affecting the population of vectors. Heavy rain, for example, can decrease vector populations of aphids or whiteflies, decreasing disease transmission. Presence or absence of wind can carry disease vectors. Temperature has a similarly strong affect on viral vectors.

Both temperature and light intensity affect the expression of resistance. Temperature higher than 30 C, for example, breaks down the resistance to tomato spot wilt virus in pepper. Conversely, CMV is more severe at lower temperatures. High light intensity generally favors the expression of resistance. Evaluation of resistance in a greenhouse with adequate temperature and light control or in an incubator with more precise environmental control would provide reliable measures of resistance.

Given that these environmental factors have such strong affects, measures of resistance are challenging to standardize. Visual ratings of disease symptoms are the only "reliable" method for evaluating resistance, as these relate directly to the health of the individual plant, without regard to viral presence.

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

   There would be little ecological impact on most wild plant populations because naturally occurring resistance is already relatively high. This is the case for wild relatives of *Cucurbita* spp., which typically grow in marginal areas or disturbed environments (e.g. roadsides, tree-fall clearings). Even a large increase in viral tolerance is not likely to have a large consequence for plant populations.

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

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

    Crop plants in commercial field are often infected with multiple viruses. For example, some pepper plants have been shown to be infected with up to 7 viruses. Recombination and possibly heterologous encapsidation are likely occurring naturally, irrespective of the presence of PVCP-PIPs. The risk of novel viral interactions involving a viral transgene in biotech plants would not be higher than that already existing in traditional crop plants. Because traditional sources of resistance to plant viruses are less effective and do not confer complete resistance, the plants still support viral populations. As a result, the overall number and volume of viral particles consumed is higher with the traditional sources of resistance. The higher viral populations in the non-resistant or partially resistant plant populations will, if anything, lead to a higher probability of transencapsidation.

    Research by Fuchs and Gonsalves with biotech squash indicated that heterologous encapsidation, but not recombination, may occur at a very low rate (2%). In another experiment, the rate was 0%. Based on their experiments, they concluded that such encapsidation was "not durable and had restricted impact on the environment." See attached report from the 8th International Symposium on the Biosafety of Genetically Modified Organisms.

The NRC Committee in its 2000 report concluded that regarding recombination between transgenes and viral pathogens, in all cases examined, only homologous sequences were exchanged. No experimental data indicate that recombination can occur between virus genomes and transgene sequences that are derived from distantly related or unrelated viruses. Since trans-encapsidation does not involve exchange of genetic material, any unique insect vectoring properties of a transencapsidated virus genome will not be inherited.

The USDA as well as Seminis' petitions also addressed this issue. Cucurbits are readily infected with multiple viruses including CMV, ZYMV and WMV2. The virus titer within multiple infected plants can be several hundred times greater than the level of the biotech RNA or coat protein in ZW-20 or CZW-3 plants. The elements contained in ZW-20 and CZW-3 do not pose an exposure of viral components since these components have had the potential to interact with one another in nature in mixed infections. The biotech lines may actually reduce the probability of recombination merely by the fact that the virus titer of CMV, ZYMV and WMV2 is significantly reduced in the biotech lines ZW-20 and CZW-3.

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

    Seminis knows of no evidence showing that the frequency of viral recombination and/or heterologous encapsidation in a biotech plant is higher or lower than that in traditionally-bred plants.

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

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

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

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

    The PVCP-PIP biotech plants do not introduce any significantly new genetic material to the environment that was not already present.

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

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

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

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

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

No. Even though there was no evidence to suggest that potyviral coat proteins are involved in synergy, Asgrow inoculated both ZW-20 and CZW-3 squash plants with several common squash-infecting viruses. Using other common cucurbit viruses, like CMV and PRV, Asgrow demonstrated that there was no synergy between the ZYMV and WMV2 transgenes and other viruses that commonly infect squash. This demonstrated that the transgenes would not result in other viruses causing severe symptoms. Although highly unlikely, if synergistic symptoms occurred in ZW-20 or CZW-3 plants, this would only be an agronomic phenomenon and have no long-term environmental impact (American Institute of Biological Sciences (AIBS). 1995. Biotech virus-resistant plants and new plant viruses. Meeting report from AIBS workshop sponsored by U.S. Department of Agriculture. 47pp).

2000 National Research Council (NRC) Report
"Genetically Modified Pest-Protected Plants: Science and Regulation"
Seminis' Response

Following are Seminis' comments on the information, analyses, and conclusions on viral coat proteins, with specific focus on Seminis' biotech virus-resistant **squash lines ZW-20 and CZW-3**

General

The NRC report presented the biotech virus-resistant squash as a case study to illustrate potential risks posed by virus-derived transgenes. In the report, the Committee concludes that USDA's assessment about how the spread of virus-protective transgenes will affect free-living *Cucurbita pepo* populations is not well supported by scientific studies, suggesting that the review by the USDA was not rigorous enough.

It should be noted that the deregulation of the biotech virus-resistant ZW-20 squash line by USDA was the result of a rigorous 2-year review by USDA, and information was gathered from an array of sources. It was supported by several years of data accumulation from initial contained tests in the greenhouse, and small-scale field trials to large-scale field trials. During the deregulation process USDA solicited public comments on the petition and the comments received were carefully considered. Furthermore, USDA consulted state extension agents in relevant states and USDA requested additional studies from Asgrow (now part of Seminis Vegetable Seeds), which were performed. All this was done in addition to the usual independent scientific literature survey and expert consultations USDA routinely does on top of the voluminous information requested of petitioners.

Health issues

The health concerns about biotech virus protected squash have been related to both viral and bacterial genes that are expressed in all the plant's cells. As mentioned in the NRC report, human and animal consumption of plants with viral coat proteins is widely considered to be safe, on the basis of common exposure to these proteins in non-biotech squash. Asgrow performed a quantitative ELISA analysis of the viral coat protein and for CZW-3 also of the linked NPT II protein. The average coat protein levels in greenhouse grown fruits of both biotech ZW-20 and CZW-3 were found to be significantly lower that viral coat proteins found in virus infected non-biotech cucurbit fruits collected from the local grocery store. In the fruit from biotech CZW-3 there were very low levels of NPT II protein. The NPT II protein is ubiquitous in the environment (Flavell *et al.*1992). The safety of NPT II was detailed in publications of Fuchs *et al.* (1993a, b), Flavell *et al.* (1992) and Nap *et al.* (1992). All data available indicate that the kanamycin resistance gene can be safely used. The FDA has approved many crops with the kanamycin resistance gene. Furthermore, FDA has given food additive status to the NPT II protein.

Environmental issues

As the NRC report indicates the major environmentally adverse effects that have been discussed in connection with virus-resistant crops pertain to effects of viral coat protein genes on the pathogenicity of other viruses and consequences of crop-to-wild gene flow

that could allow beneficial transgenes to move into feral crop plants or closely related weeds.

Putative effects of viral coat protein genes on the pathogenicity of other viruses.

The first issue was studied experimentally and it was concluded that the risks that other viruses would become transmissible (from heteroencapsidation) or that the non-pathogenic viruses would become more virulent (from recombination) were exceedingly small (Fuchs *et al.* 1998).

The Committee concludes that regarding recombination between transgenes and viral pathogens, in all cases examined, only homologous sequences were exchanged. No experimental data indicate that recombination can occur between virus genomes and transgene sequences that are derived from distantly related or unrelated viruses. Regarding transencapsidation and gain-of-transmission characters, it is highly unlikely that functional coat proteins expressed in biotech plants pose a significant risk of expansion of host range to new crop or non-crop hosts. Transencapsidation does not involve exchange of genetic material, meaning that any unique insect vectoring properties of a transencapsidated virus genome will not be inherited. No data indicate that expression of viral coat proteins enhance the virulence of heterologous viruses. Studies by Fuchs & Gonsalves show that heteroencapsidation will not cause epidemics and this is not likely to have significant consequences to the environment.

Asgrow addressed the concerns about the squash also posing the risk that its virus genes or the coat proteins they produced might interact with other viruses to produce new viruses by conducting a study on mixed infections of ZW-20. Using other common cucurbit viruses, like CMV and PRV, Asgrow demonstrated that there was no synergy between the ZYMV and WMV2 transgenes and other viruses that commonly infect squash. This demonstrated that the transgenes would not result in other viruses causing severe symptoms.

USDA and the petition also addressed this issue. Cucurbits are readily infected with multiple viruses including ZYMV and WMV2. The virus titer within multiple infected plants can be several hundred times greater than the level of the biotech RNA or coat protein in ZW-20. The elements contained in ZW-20 do not pose an exposure of viral components since these components have had the potential to interact with one another in nature in mixed infections. The biotech line may actually reduce the probability of recombination merely by the fact that the virus titer of ZYMV and WMV2 is significantly reduced in the biotech line ZW-20.

The second issue, whether (wild) relatives could benefit from viral coat protein genes was according to the NRC report more controversial.

Crop-to-crop gene flow

The genus *Cucurbita* includes five domesticated species and 22 wild species (Decker 1988). The common ancestor of all cucurbits is probably an annual gourd-producing plant that was first used in the New World agriculture about 10,000 years ago. The *C. pepo* lineage appears to be composed of two subsets, formally identified as two subspecies *ovifera* and *pepo*. Subspecies *pepo* includes domesticated types, pumpkins, zucchini, marrow varieties, and some ornamental gourds, whereas subspecies *ovifera* var. *ovifera* includes the remaining ornamental gourds varieties and acorn, crookneck,

straightneck, scallop and yellow squash (Wilson 1993). The five cultivated cucurbit species include *C. pepo*, *C. maxima*, *C. mixta*, and *C. moschata*. The fifth species is *C. ficifolia*, which is mostly cultivated in South America. Inter-specific hybridization has been extensively investigated and is well understood in the four first-mentioned cultivated species. F_1 hybrids can be obtained in breeding programs, but only with difficulty and such hybrids usually are sterile. There is no evidence of spontaneous hybridization among these four species despite the fact that they have been grown side by side under cultivation for many generations (Whitaker & Robinson 1986).

A weed pest is a plant that grows persistently in locations where it is unwanted. There are several definitions of weediness, but they all have the undesirable nature of weeds from the point of view of humans in common (de Wet & Harlan 1975). Significant differences were found in the distribution of weedy characteristics among weeds, 'normal plants' and crops (Baker 1965; Keeler 1989). Baker (1965) described 12 common weed attributes, which include rapid growth to germination and flowering in many environments, internally controlled discontinuous germination, long-lived seeds, continuous seed production, use of wind or unspecialized insects for pollination if outcrossing occurs, high seed production and good competitiveness. Baker's list of weed attributes can be used as an imperfect guide to the likelihood that a plant will behave as a weed. Keeler (1989) analyzed *C. maxima*, a close relative to *C. pepo*, and stated that *C. maxima* possess 3 out of 15 characteristics of plants that are notably successful weeds. Those characteristics are: continuous production of seeds as long as growing conditions permits, use of unspecialized insects as pollinators, and strong competitiveness with other plants. However, *C. pepo* that has been bred for agricultural use, e.g. yellow crookneck squash, has few traits that are associated with weediness. However, despite the extensive cultivation of *C. pepo* in the US and Mexico since antiquity, there is no body of scientific reports of significant weediness of *C. pepo* in those countries. *C. pepo*, yellow crookneck squash is not listed as weed in the Federal Noxious Weed Act (7 U.S.C. 2801-2813) (see Westbrooks 1998) and is not reported by the Weed Society of America to be a common or troublesome weed anywhere in the US (Holm *et al.* 1979; Muenscher 1980). Also in the NRC report it is mentioned that volunteer squash plants are not known to spread and become weeds. Over-wintering volunteers are always killed after germination by spring frosts, as squash is very sensitive to cold. The Committee stated that it knows of no scientific evidence that crop-to-crop gene flow has caused health or environmental risks to date. Contamination with pollen from other farms is likely to be very low in most cases. Based on traditional breeding practices, isolation distances has been established for a range of (outcrossing) species, which can also be used as reference for the isolation of biotech (squash) crops.

Crop-to-wild gene flow

Interspecific hybridization has been extensively investigated. In the United States, there exist three free-living subspecies of *C. pepo* (FLCP) that can cross with cultivated varieties of *C. pepo* without loss of fertility (Whitaker & Bemis 1964; Nee 1990). These include the free-living gourds in Texas designated *C. pepo* ssp*. ovifera* var*. texana* and free-living gourds in Illinois, Arkansas and Oklahoma designated *C. pepo* ssp*. ovifera* var*. ozarkana* (Wilson 1993) and a putative relative of *C. pepo* that was rediscovered at several sites in northeastern Mexico during the early 1980's, designated *C. pepo* ssp. *fraterna* (Nee 1990). Both ssp. *ovifera* can cross with cultivated *C. pepo* varieties through natural pollination mediated by honey bees without loss of fertility (Kirkpatrick *et al.* 1985). Field experiments done by Fuchs & Gonsalves showed that the coat protein genes of CMV, ZYMV and WMV2 could readily move from biotech squash into *C. texana*

upon hybridization and introgression. Movement of the coat protein genes provided resistance against these three viruses to *C. texana* and a selective advantage under conditions of high virus pressure in contrast to under low virus pressure where there is no selective advantage. However, under high virus pressure, transfer of transgenes to *C. texana* virtually did not occur because the *C. texana* plants severely affected by the virus and produced very few flowers.

C. texana is not a noxious weed. The question is, would it become a noxious weed if it became virus-resistant? Free living squash populations (FLCP) are not reported to be a serious problem in unmanaged or agriculture ecosystems. Squash is not listed as a weed in the Federal Noxious Weed Act (7 U.S.C. 2801-2813) and is not reported by the Weed Society of America to be a common or troublesome weed in the U.S. (Bridges & Baumman 1992). FLCP have previously been listed as a significant weed in soybean and cotton fields in Arkansas. The Arkansas representative to the Weed Society of America, Dr. Baldwin stated that FLCP are not currently as significant a problem in this region as they were in the past. Dr. Weidemann from the Univ. of Arkansas, who conducted research to identify biological control agents to eliminate FLCP from soybean fields, confirmed that FLCP are only a minor problem in Arkansas in recent years because FLCP have been controlled through the use of new herbicides. As mentioned in the NRC report, in 1977 FLCP were listed as one of the top-10 most important weeds in Arkansas.

The critical question was whether viruses kept the population of wild squash, which produces inedible gourds, in check. In other words, if gene flow occurs between VR biotech and wild relatives, will the VR gene be retained? Asgrow conducted a survey to test this. The evidence from the virus survey of wild populations indicated that there is not continuous virus pressure on these wild relatives and under those circumstances the virus resistance gene will not have any selective advantage over plants that lack this gene.

Some experts in biosafety research said the study of 14 plants from 9 locations was too limited to draw valuable conclusions. However, the survey did not represent a mere 14 plants, but 9 populations of FLCP, each population consisting of many individuals. It was based on extension agents visits to 9 sites in Mississippi, Louisiana, and Arkansas with instructions to survey the populations for the presence of virus symptoms, which would readily be visible on FLCP. If the extension agents saw symptoms, they were to take leaf samples for Asgrow to analyze. However, no visible symptoms were observed in any of the locations. As an extra precaution, our collectors were asked to collect a representative vine to double check through ELISA, double diffusion serology and inoculations onto indicator plants in order to confirm and validate visual evaluation. Field experiments done by Fuchs & Gonsalves indeed showed that under conditions of low virus pressure, hybrids that expressed the three CP genes did not appear to have any selective advantage over *C. texana* and their non-biotech counterparts. Furthermore, preliminary survey by Fuchs & Gonsalves indicated that *C. texana* are not readily infected by viruses in their natural ecosystems. Therefore, the selective pressure to maintain the virus resistance trait in the wild population will be minimal. In conclusion, based on field experiments, free-living *C. texana* resistant to ZYMV, CMV and WMV2 are unlikely to become a significant threat to the environment as invasive and eventually even more noxious weed pests.

The concern regarding the risk of biotech virus resistance causing wild relatives to become more weedy seems to ignore the fact that resistance genes to these viruses

already exist in *C. pepo* and can possibly cross into wild relatives. Thus, the biotech virus-resistant squash does not contain traits that are not already present in the germplasm pool. Zucchini squash with resistance to CMV have been available from seed companies for many years. Traditionally bred varieties with resistance to ZYMV and WMV are also commercially available. There are also other species of wild squash, which are present in the US that are resistant to PRV, CMV and WMV. Despite the fact that they have these resistances, these species are not known to be significant weed problems in the US.

In the report, commissioned by APHIS, Wilson, a squash expert at Texas A&M Univ. was asked "if crop/weed interaction has occurred within the *C. pepo* complex of domesticated and free-living forms throughout the 3,000 year history of human agricultural activity in the Eastern US, then why be concerned about possible involvement of transgenic strains?" All available evidence, both archaeological and botanical, indicates that new, domesticated elements of *C. pepo* complex have been sequentially introduced into the agricultural systems of eastern North America over the past 3,000 to 7,000 years. Wilson claims that the source of transgenes, and unknown interactions between these unique genetic elements, which are not part of the *C. pepo* complex, and the *C. pepo* genome represent an unknown and untested factor. The process of injecting a foreign genetic element, a functional gene that has no precedent within the phylogenetic history of the crop/weed system, constitutes a biological risk. This reasoning seems to overlook the fact that resistance genes to these viruses already exist in *C. pepo*. Traditionally bred varieties with resistance to ZYMV, CMV and WMV are commercially available and there are species of wild squash carrying resistance to PRV, CMV and WMV. As said earlier the biotech virus-resistant squash does not contain traits that are not already present in the germplasm pool. The introduced CMV, WMV2 and ZYMV coat protein genes encode viral coat proteins substantially similar to viral coat proteins found in abundance in virus infected traditionally bred squash fruits as was found when viral coat protein levels were determined in non-biotech squash fruits collected from local grocery stores. No convincing data were generated that backed Dr. Wilson's statement that data presented in his report point towards the clear presence of risk regarding increased weediness and loss of crop plant biodiversity. The 'africanized honey bee' which Dr. Wilson mentions as example of the impact on natural populations due to both intentional and accidental human manipulations is not relevant for the squash case. Most if not all cases in which human intervention has caused adverse ecological effects refer to introduction of exotics, like the rabbits and Opuntia cactus in Australia. Placing virus-resistant squash in its natural habitat will not have such drastic effects. Dr. Wilson's concerns about the introduction of biotech virus-resistant squash are not backed by other prominent scientists in the field including Drs. Provvidenti and Robinson of Cornell University, who are both world renown in the area of breeding and pathology in Cucurbits. They supported the release of the virus-resistant biotech squash.

Exemption of viral coat proteins

In addition to exempting plant-pesticides derived from sexually compatible plants, the 1994 and 1997 EPA documents propose a number of more specific exemptions. EPA generally provides more reasonable scientific justification for these exemptions. One specific class of plant products that was proposed for categorical exemption was viral coat proteins. Viral coat proteins are already present in foods because of natural virus infections of crops and have not caused obvious medical problems, so health concerns are considered minimal. The EPA exemption of viral coat proteins is also based on considerations that "include the low potential for adverse effects to non-target organisms

and the potential benefits (environmental and economic) of utilizing virus coat protein mediated resistance." The NRC committee, in general, agrees with this assessment of the minimal health and non-target effects posed by viral coat protein expression in crop plants and concludes that “Viral coat proteins in biotech pest-protected plants are not expected to jeopardize human health because consumers already ingest these compounds in non-biotech food. However, the committee questions the categorical exemption of all viral coat proteins under FIFRA due to concerns about outcrossing with weedy relatives”.

In the report, it is stated that although ecological concerns are discussed and a more restrictive exemption that considers outcrossing is presented, the proposed rule favors complete exemption of viral coat proteins. It is not completely clear what is meant by the proposed rule because the Committee advises that “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, in which the Agency can limit its exemption considering the gene transfer risks associated with the introduction of viral coat proteins to plants”. Following the decision trees for viral coat proteins in virus-resistant squash for health and weedy-relative concerns as given in paragraphs 3.3.1 and 3.3.2 of the NRC report, in both cases lead to exemption as indicated hereunder.

Health concern:

1) Is the substance found in plant parts that consumers, including human and non-human consumers, such as food animals or pets eat or workers come into contact with?
a) Yes or Unknown—go to 2
b) No—exempt from health concerns

2) Is the substance known to have general chemical and physical properties common to many allergens?
a) Yes or Unknown—subject to safety assessment
b) No—go to 3

3) Is the substance similar to substances that people now eat or come into contact with, and can confident predictions of safety based on the similarities be made?
a) Yes—go to 4
b) No or Unknown—subject to safety assessment

4) Is the expected exposure to the substance substantially greater than current exposures?
a) Yes or Unknown—subject to safety assessment
b) No—go to 5

5) Is there a reasonable chance, based on known properties of the substances, that its production will lead to harmful concentrations of toxicants or allergens that consumers eat or workers come into contact with?
a) Yes or Unknown—subject to safety assessment
b) No—exempt from health concerns

Ecological concern:
1) Does the cultivated plant occur in feral populations or hybridize with related species in the United States?
a) Yes or More data needed—go to 2

b) No—exempt from weedy-relative considerations

2) Have feral populations or wild relatives been reported as weedy or invasive in the United States or have a reasonable potential to become weedy?
a) Yes or More data needed—go to 3
b) No—exempt from weedy-relative considerations

3) Does the gene for resistance confers a specific type of resistance or a greatly enhanced degree of resistance that is not found in feral populations or sexually compatible wild relatives in the United States?
a) Yes or More data needed—go to 4
b) No—exempt from weedy-relative considerations

4) Is it reasonable to expect that this trait could have a substantial impact on the population dynamics of feral plants or wild relatives and will lead to increased abundance?
a) Yes or More data needed—subject to weedy-relative considerations
b) No—Exempt from weedy-relative considerations

Conclusion

In a New York Times article of Nov. 3 1999, a squash grower was interviewed. He indicated that viruses devastating his squash plants, giving no yield and, besides breeding virus-resistant varieties either through biotech or traditional breeding, there is no cure to this disease. Therefore, the biotech virus-resistant squash plants, which show very high protection, are especially valuable in light of the difficulties to obtain similar multiple resistance by traditional breeding strategies or to control vector populations by cultural practices and the use of chemicals.

In summary, the NRC report provides no compelling reason for the withdrawal of the decision to consider Seminis' ZW-20 and CZW-3 biotech virus-resistant squash non-regulated products. The report suggest further areas of study regarding the putative outcrossing from cultivated *C. pepo* into free living *C. pepo* subspecies. Such further research has been done or is taking place in public research centers, (partly) funded by USDA grants, like the 3-year study taking place at the USDA Agricultural Research Service, Madison (WI) about the gene flow from biotech *C. pepo* into free living population by Staub, Quemada & Walters and the 3-year study of Falk (UC, Davis) on the incidence and origin of new viruses in multiple virus-resistant Cucurbits. Additional work on the area of outcrossing or recombination continues to support the finding of the USDA determination. Additionally, these studies reflect a desire to maintain a stewardship component for this technology. Seminis have provided samples to university researchers funded by USDA to assist in such stewardship research.

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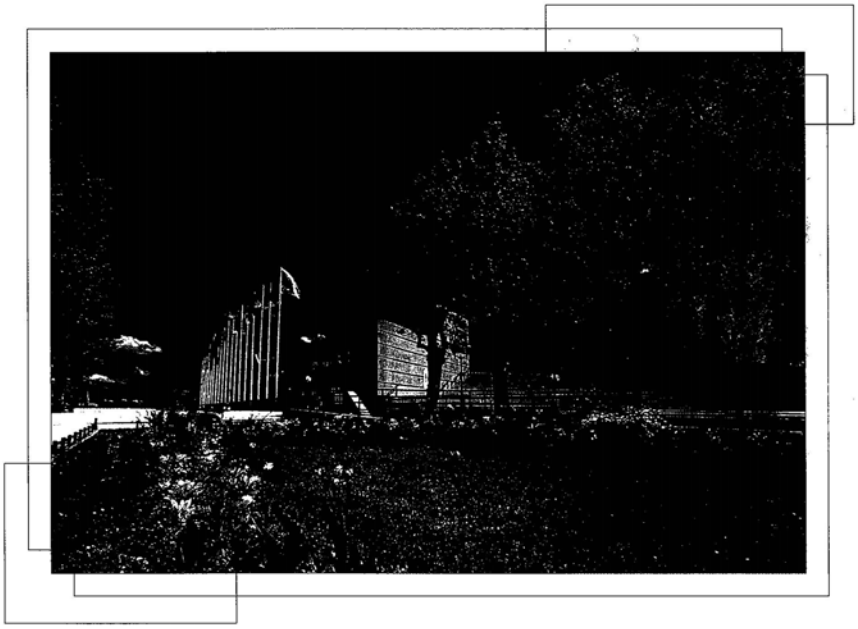

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Environmental safety assessment of virus-resistant transgenic squash: Lessons and perspectives

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Virus-resistant transgenic squash cultivars are commercialized in the United States ever since the deregulation of transgenic crookneck squash lines ZW-20 and CZW-3 in 1994 and 1996, respectively. Line ZW-20 is expressing the coat protein (CP) genes of *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus* (WMV), and is resistant to these two viruses (1, 2, 3, 4), whereas line CZW-3 is expressing the CP genes of ZYMV, WMV, and *Cucumber mosaic virus* (CMV) and is resistant to these three viruses (2, 3, 4).

Environmental safety issues have been raised on the use of virus-resistant transgenic crops, including squash, due to the occurrence and consequences of heterologous encapsidation, recombination, and gene flow (5). Heterologous encapsidation and recombination can generate viruses with altered properties, including expanded host range, increased pathogenicity, and changes in vector specificity, and gene flow can turn wild plant species into noxious weeds in agricultural fields and natural habitats (5). We used various virus-resistant transgenic squash lines to assess these issues under field conditions.

Heterologous encapsidation and recombination were assessed by monitoring the spread of the aphid nontransmissible strain MV of ZYMV in field plots of conventional and transgenic squash expressing the CP gene of an aphid transmissible strain of WMV (6). The experimental approach was to mechanically inoculate transgenic squash with ZYMV strain MV and monitor subsequent aphid-mediated transmission, assumed to be achieved either through heterologous encapsidation and/or recombination, to uninoculated squash. Spread of ZYMV strain MV occurred at a very low rate (2%), most likely through heterologous encapsidation but not recombination, in fields of transgenic but not conventional squash (6). No epidemic of ZYMV strain MV developed over two consecutive years, even under conditions of high disease pressure and low selection pressure against recombinant viruses (6). In contrast, spread of the aphid transmissible ZYMV strain NY, which was used as control, readily occurred (99%) (6). Since heterologous encapsidation does not modify the viral genome, the limited phenotypic alterations observed in the case of ZYMV strain MV were not durable and had restricted impact on the environment. The same experimental approach was used to assess the spread of the aphid nontransmissible strain C of CMV in fields of transgenic squash expressing the CP gene of an aphid transmissible strain of CMV. Interestingly, spread of CMV strain C did not occur to detectable levels over two consecutive years (7).

Pollen-mediated gene flow from transgenic squash CZW-3 into the wild relative *Cucurbita pepo* spp. *ovifera* var. *texana* was also assessed (8, 9) Transgene dissemination readily occurred from transgenic F1 hybrids into C. *texana* over three generations in field settings where test plants grew sympatrically and viruses were not limiting the growth, and fruit and seed production of C. *texana* (8) In contrast, introgression was not sustained under conditions of high viral disease pressure (8). Fitness evaluation studies indicated that back cross C. *texana* progeny that acquired the CP transgenes upon hybridization and introgression not only were resistant to CMV, ZYMV, and WMV, but also grew more vigorously, and produced a greater number of mature fruits and viable seeds than C. *texana* under conditions of high disease pressure (9). In contrast, under conditions of low disease pressure, C. *texana* outperformed transgenic and nontransgenic segregants (9). These results clearly demonstrate that C. *texana* hybrids with CP transgenes could have a selective advantage if CMV, ZYMV, and WMV are severely limiting the growth and reproductibility of wild squash populations. Interestingly, a very low incidence of viruses (0-0.75%) has been consistently detected in wild squash populations assayed so far for the presence of the four major cucurbit viruses (10). Thus, there is little, if any, evidence that the introduction of virus resistance could provide wild squash species with a tremendous selective advantage.

It is important to highlight that heterologous encapsidation and recombination are environmental safety issues that apply not only to transgenic crops expressing virus-derived gene constructs but also to conventional crops subjected to mixed virus infection. Thus, a critical issue is to determine whether virus-resistant transgenic crops pose any increased risk beyond natural background events. Similarly, regarding gene flow, limited, if any, difference is expected between genetically engineered virus resistance and virus resistance bred by conventional techniques because it is the resistance trait that is being scrutinized, regardless of the crop development strategy.

Our field safety assessment studies with virus-resistant transgenic squash have provided new insights relevant to agricultural practice and environmental impact. Thus, our data are useful to policy makers for taking scientifically-based decisions on the safe release of virus-resistant transgenic crops. Also, our studies are contributing to promote open, informed, and pro-active dialogues within the scientific community, and between the scientific community and organizations engaged in formulating issues, opinions, and recommendations on plant health and sustainable agriculture. We hope our studies will help increase the acceptance of virus-resistant transgenic crops across borders and continents, and facilitate technology transfer since our field data clearly show so far that benefits of virus-resistant transgenic crops outweigh by far risks to the environment (1, 2, 6, 7, 8, 9, 11, 12, 13).

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