Comments on the human health and product characterization sections of EPA's *Bt* Plant-Pesticides Biopesticides Registration Action Document

By
Michael Hansen, Ph.D.
Consumer Policy Institute/Consumers Union

Presented to the EPA Science Advisory Panel
Arlington, VA
October 20, 2000

Thank you for the opportunity to present the comments of the Consumer Policy Institute/Consumers Union[1] on a subset of the Environmental Protection Agency's *Bt* Plant-Pesticides Biopesticides Registration Action Document. We would like to comment on two questions: human health questions and product characterization. We believe that the human health data that EPA presently requires are inadequate and that more data need to be taken. Below, we propose some of the additional data that should be required before any of these crops can be reregistered.

Please provide comment on whether the human health data is an adequate evaluation of the risk from the Bt proteins. What, if any, additional data is necessary to assess the risk from the Bt-expressing plant-pesticide products?

We would particularly like to address the issues of allergenicity, antibiotic resistance marker genes and acute toxicity testing.

Allergenicity

[1] The Consumer Policy Institute is a division of Consumers Union, publisher of *Consumer Reports* magazine. Consumers Union is a nonprofit membership organization chartered in 1936 under the laws of the State of New York to provide consumers with information, education and counsel about goods, services, health, and personal finances; and to initiate and cooperate with individual and group efforts to maintain and enhance the quality of life for consumers. Consumers Union's income is derived solely from the sale of *Consumer Reports*, its other publications and from noncommercial contributions, grants and fees. In addition to reports on Consumers Union's own product testing, *Consumer Reports*, with approximately 4.5 million paid circulation, regularly carries articles on health, product safety, marketplace economics and legislative, judicial and regulatory actions that affect consumer welfare. Consumers Union's publications carry no advertising and receive no commercial support.

We do not think that the human health data that EPA currently has are adequate. In particular, EPA seems to have ignored a crucial study that suggests that the *Bt* delta-endotoxin is an inhalant allergen, which could present risks, in an occupational sense, to farmworkers and millworkers that are exposed to dust from the processing of *Bt* crops.

EPA maintains that "After decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports of immediate or delayed allergic reactions to the delta-endotoxin itself despite significant oral, dermal and inhalation exposure to the microbial product" (EPA, 2000: pg. IIB6). We believe that this statement is at best misleading. Last year, an EPA-funded study was published in *Environmental Health Perspectives* titled "Immune responses in farm workers after exposure to *Bacillus thuringiensis* pesticides." The article pointed out that more work needs to be done to evaluate the allergenic potential of *Bt* sprays, as there have been 3 studies that are suggestive of the *Bt* sprays having an effect. The authors are concerned because, as they point out, "approximately 75 percent of asthma cases are triggered by allergens and morbidity and mortality due to asthma have increased considerably over the past 20 years" (Bernstein et al, 1999: 570).

The study consisted of a surveillance program of farm workers before and after exposure to *Bt* pesticides. This study does not show a definitive link between exposure to *Bt* sprays and occupationally related respiratory symptoms, but did find that a number of the workers exhibited skin sensitization and presence of IgE and IgG antibodies with those responses being more numerous in those workers with higher levels of exposure. Both skin sensitization and IgE antibodies are components of an allergic response.

As part of the study, the scientists were able to show that 2 of the farm workers studied had a positive skin-prick test to the Btk spore extract containing the pro-delta-endotoxin active component. This means that there are now skin and serologic agents that could be used to test the potential allergenicity of the various delta-endotoxins that have been engineered into *Bt* crops. Although the authors say that their results should allay some of the concerns about the allergenicity of transgenic foods from *Bt* crops, they clearly say that they now have the skin and serologic agents to do such tests: "Because reactivity to the *Btk* pro-delta-endotoxin was only encountered in 2 of 123 workers sensitized by the respiratory route, it is unlikely that consumers would develop allergic sensitivity after oral exposure to transgenic foods (e.g., tomatoes, potatoes) that currently contain the gene encoding this protein. *However, future clinical assessment of this possibility is now feasible because of the availability of reliable Bt skin and serologic reagents developed during the course of this investigation"* italics added (Bernstein et al., 1999: 581).

Given that such *Btk* skin and serologic agents exist, we feel that all the Bt crops should be retested using these skin and serologic reagents. In particular, EPA should not reregister the *Bt* corn varieties until this allergy testing has been done.

Although the reaction was to the pro-delta-endotoxin, separate genetic studies (gel electrophoresis and hybridized blot analysis) demonstrated the presence of genes for the Cry 1Ab and Cry1Ac delta endotoxins in both spray formulations (Javelin and Agree) to which the workers had been exposed. Truncated version of the Cry1Ab and Cry1Ac are present in the Bt corn and Bt cotton events, respectively. Unless the allergenic epitopes are all found in the part of the delta endotoxin that is removed during truncation, one could reasonably expect that the *Bt* corn and *Bt* cotton crops would contain an allergenic epitope. Furthermore, use of these reagents would be superior to the current criteria presently used to evaluate the allergenicity of these crops: amino acid sequence homology to known allergens; resistance to acid and gastric digestion; heat stability/heat resistance; and molecular size. None of these criteria are exact as the state of science in the field of allergenicity is still in its infant stages. (SAP, 2000: 7; www.epa.gov/scipoly/sap/2000/february/foodal.pdf).

Clearly, if skin and serologic reagents from humans exist for a given protein, then any allergenicity testing must use such reagents. If the reagents become available after the crops have been approved, EPA should require the companies to retest those crops because the human reagents are far more accurate than the four criteria presently being used.

Since one of the co-authors of the paper, Dr. Donald Doerfler, is an EPA scientist, we wonder why the EPA hasn't already moved to conduct these tests. With the use of these skin and serologic reagents, the testing of *Bt* crops would not take that long and would be relatively inexpensive. So why hasn't such a test been carried out?

EPA has argued that there occupational exposure to the Cry9C protein (or the other Cry proteins inserted into corn, cotton) is negligible, or presents no risk because the Cry proteins are not toxic to people (Biopesticide Fact Sheet, 1999). Yet the EPA presented no study to substantiate the claim of negligible exposure.

In fact, there is scientific evidence that occupational exposure to grain dust can lead to allergic symptoms, with the classic case being bakers' asthma (Baur, 1998). Recent studies have also implicated corn dust in respiratory dsyfunctions including acute respiratory inflammation (Park et al., 1998; Wohlford-Lenane et al., 1999) and in glove-lubricant-powder derived allergy (Crippa et al., 1997). Thus, corn dust can clearly convey allergens, and the pro-delta-endotoxin is potentially allergenic, so there is ample evidence to be concerned about occupational exposure to grain dusts, especially corn.

Interestingly, while the authors of that study found that farm workers had skin reactions and IgE antibodies to *Bt* spray, they could not link any respiratory symptoms to the occupational exposure. However, this could be a result of the fairly low levels of *Bt* that the farm workers were exposed to. The concentration of delta-endotoxin in the *Bt* crops, particularly corn, is between one to two orders of magnitude higher compared to *Bt* sprays. That's why the insect resistance management strategy is called the "high dose" strategy. Furthermore, the concentration of the Cry9C protein in the seed is one to two orders of magnitude higher than the concentration of Cry1Ab or Cry1Ac in corn and cotton, respectively—18.6 μg/gm (kernal) for Cry9C vs. 1.4 μg/gm (kernel), 0.19-0.39 μg/g (grain), and 1.62 μg/g for Cry1Ab-*Bt*11, Cry1Ab-MON810, and Cry1Ac, respectively (EPA, 2000: pg. IIC17). So, the concentration of Cry9C in corn dust could conceivably be 2 to 3 orders of magnitude higher than they level of endotoxin found in foliar Bt sprays.

So, the Bt crops have far higher levels of endotoxin in the grain and leaves than do the foliar Bt sprays. Furthermore, while farm workers are exposed to the foliar Bt sprays, workers in mills or other areas where grains are being processed would be exposed to grain dust and so could conceivably be exposed to far higher quantities of the *Bt* endotoxin than a farm worker would.

Antibiotic resistance marker genes

In 1991-1992, when FDA was developing its policy of GE plants, the conventional wisdom in the scientific community was that DNA was a very fragile molecule that would be readily broken down in the environment and would not survive digestion in the gut. We now know that both assumptions may not always be valid (Traavik, 1998). Even though DNases (molecules that break down DNA) are widely distributed in the environment, free DNA has been found in all ecosystems (marine, fresh water, sediments) studied (Lorenz and Wackernagel, 1994). Indeed, pooled data suggest that free DNA is present in significant amounts in the environment. Larger amounts of DNA are extracted from soil than can be extracted from the cells in the soil (Steffan et al., 1988). Further studies have shown that this free DNA in the soil comes from microorganisms that no longer occur in that habitat (Spring et al., 1992) thus demonstrating that DNA can out-survive the organism it came from and still be capable of being taken up and expressed by microorganisms. Finally, yet other studies have found that pollution (i.e. xenobiotics) can affect the survivability of DNA and the possibility of its transfer to other organisms (Traavik, 1998).

These data lead to serious concerns about the antibiotic resistance marker genes that are present in virtually all engineered plants presently on the market. These genes code for proteins that confer resistance to a given antibiotic. The possibility therefore exists that these genes for antibiotic resistance could be taken up by bacteria, thus

exacerbating the already very serious problem of antibiotic resistance in disease causing organisms.

In mammalian system, the question is whether foreign DNA can survive digestion, be taken up through the epithelial surfaces of the gastrointestinal or respiratory tract or not, or be excreted in feces. Studies in the 1970s (Maturin and Curtiss, 1977) and 1980s (McAllan, 1982) in rats and ruminants, respectively, suggested that nucleic acids (e.g. DNA and RNA) failed to find evidence that DNA survived digestion. Consequently, many scientists assumed that DNA was readily digested. However, the methods used to detect DNA were not very sensitive. In the mid-1990s, researchers in Germany, re-investigated the issue, using far more sensitive methods (Schubbert et al., 1994). Mice were fed DNA from the M13 bacteriophage either by pipette or by adding it to the feed pellets. Using sensitive hybridization methods and PCR (polymerase chain reaction) the authors found 2-4% of the M13 DNA in feces and 0.01-0.1% in the blood—both in serum and cell fraction. Sizeable DNA fragments (almost a quarter of the M13 genome) could be found up to 7 hours after uptake.

If free DNA is not immediately digested in the gastrointestinal tract, the possibility also exists that it can be transferred to bacteria that live there. A recent study utilizing a simulated human gut demonstrated that naked DNA had a half-life of 6 minutes, more than enough time for such DNA to transform bacteria (ref to come).

In another experiment, a genetically engineered plasmid was found to survive (6 to 25%) up to an hour of exposure to human saliva (Mercer et al., 1999). Partially degraded plasmid DNA also successfully transformed *Streptococcus gordonii*, a bacteria that normally lives in the human mouth and pharynx although the frequency of transformation dropped exponentially with time. Transformation occurred with either filter-sterilized human saliva or unfiltered saliva. The study also found that human saliva contains factors that increase the ability of resident bacteria to become transformed by “naked” DNA. Since transgenic DNA from food is highly unlike to be completely broken down in the mouth, it may be able to transform resident bacteria. Of particular concern would be the uptake of transgenic DNA containing antibiotic resistance marker genes, which are found the majority of GE crops presently on the market. It should be pointed out that the antibiotic marker gene present in Novartis’ Bt corn, which codes for resistance to ampicillin, is under the control of a bacterial promoter rather than a plant promoter which would further increase the possibility of expression of the ampicillin resistance gene if it were taken up by bacteria.

In September, 1998, the British Royal Society put out a report on genetic engineering that called for ending the use of antibiotic resistance marker genes in engineered food products (Anonymous, 1998). In May, 1999, the British Medical Association, which represents some 85% of the doctors in Britain, released a report calling, in part, for a prohibition on the use of antibiotic resistance mark genes in genetically engineered

plants: "The BMA believes that the use of antibiotic resistance marker genes in GM foodstuffs is a completely unacceptable risk, however slight, to human health. . . Recommendations . . . 6. There should be a ban on the use of antibiotic resistance marker genes in GM food" (BMA, 1999).

In the European Union Directive 90/220/EEC deals with the deliberate release of GEFs into the environment. The European Commission is in the process of coming with a revised version of the Directive. This revised version contains a provision which would phase out the use of antibiotic resistance marker genes by 2005 (European Commission Services, 2000)

We therefore urge EPA to prohibit use of antibiotic resistance marker genes as there is no consumer benefit for the presence of such genes in engineered foods and a potential risk.

Acute toxicity testing

At present, EPA requires only an acute toxicity feeding test. Furthermore, the delta-endotoxin that is used in these feeding tests come from a genetically engineered bacteria rather than from the transgenic plant itself. The EPA assumes that there are no real difference between a delta-endotoxin produced by an engineered bacteria and one produced in a plant.

We believe this ignores the phenomenon of post-translational processing, which consists of the modification of a protein after it has been translated from the genetic message. And such post-translational processing can have a significant impact on the structure and function of a gene. Furthermore, post-translational processing can differ between organisms, so that the same gene expressed in different genetic backgrounds may have the same amino acid sequence but may differ in structure and function. Examples of such processing includes glycosylation and acetylation.

Glycosylation consists of the addition of sugar groups (usually oligosaccharides) and can dramatically affect the three-dimensional structure and thus, function of a protein. Indeed, glycosylation is thought to be connected to allergenic and immunogenic responses (Benjuoad et al., 1992). The data presented to the EPA suggest that the delta-endotoxins are not glycosylated in the plants.

Acetylation of proteins consists of the addition of acetyl groups to certain amino acids, thereby modifying their behavior. Although incompletely understood, acetylation of the amino acid lysine has been most studied in certain groups of proteins that bind with DNA—histones and high-mobility group proteins—and such acetylation appears to be involved with the regulation of interaction of these proteins with

negatively charged DNA molecules (Csordas, 1990). However, it has been discovered that some the lysine residues in rbGH are acetylated, to form epsilon-*N*-acetyllysine when it is produced in *E. coli*. Harbour et al. (1992) found this to occur at lysine residues 157, 167, 171 and 180 or rbGH, while Violand et al. (1994) found it at residues 144, 157, and 167. The creation of this mutant amino acid may be overlooked because "(T)he identification of this amino acid cannot be determined by simple amino acid analysis because the acetyl group is labile to the acidic or basic conditions normally used for hydrolysis" (Violand et al, 1994: 1089). The effect this has on the safety, structure and function of rbGH is not known as it hasn't been actively studied.

The differences in glycosylation and acetylation that can happen when transgenes are expressed in plants or bacteria can possibly affect toxicity and therefore lend further support to the need for toxicity testing using the whole engineered food. Even if there are no differences in glycosylation (as appears to be the case for the delta-endotoxins), acetylation of lysine residue(s) could cause differences. The presence of such mutant lysine residues could easily be missed as routine amino acid analysis will remove the acetyl group; to find if there are mutant lysine residues, one must specifically look produce the transgene of interest (gene for herbicide tolerance or Bt endotoxin, for example). **Thus, whenever possible, EPA should require the companies to use material derived from the transgenic plants themselves in toxicity studies rather than bacterially-derived proteins.**

Product Characterization

Please provide comment on the quality and thoroughness of the product characterization review. What additional data, if any, should be evaluated in order to adequately characterize the Bt-expressing plant-pesticide products?

Information has appeared in the scientific literature related to the safety of foods derived from genetically engineered (GE) plants which collectively suggests that the EPA's present regulatory approach is insufficient to ensure that foods from Bt crops not pose health risks to those who consume it. This information relates to unexpected and unpredicted effects of gene insertions, and instability of the genetic characteristics that are introduced. This information leads to the view that EPA must scrutinize genetically engineered foods more closely than it has so far, and in particular should require long-terms (one to two year) animal feeding studies of the whole engineered food. Requiring a more detailed molecular characterization for each genetic transformation event will also help EPA evaluate the potential for risk and may provide a means for EPA to decide how much additional testing is needed. At present the level of molecular characterization data required by the EPA is very inadequate.

The studies which lead to greater concern about unexpected effects can be put into two categories: unpredictability of the location and expression of transgenic DNA inserts; and differences resulting from post-translational processing (e.g. proteins from the same gene are not identical in differing organisms).

Unpredictability of the location and expression of transgenic DNA underlines need for long-term toxicity tests of engineered food.

The process of insertion of genetic material via GE is unpredictable with regard to a number of parameters, including: the number of inserts of transgenic DNA, their location (chromosome, chloroplast, mitochondria), their precise position (i.e. where and on which chromosome), their structure, and their functional and structural stability. While all of these parameters can have consequences, perhaps the most important is the random or semi-random nature of the physical location of the genetic insert. The inability to control where the insertion happens is of key importance. This means that each transformation event is unique and cannot be replicated because the precise location of the insertion of genetic material always will be different.

The variable insertion site can have a number of unpredictable, and potentially negative, consequences (Doerfler et al., 1997). The insertion site can affect expression of the inserted transgene itself as well as the expression of host genes (i.e. genes in the recipient organisms). The former is known as the "position effect". A classic example involved attempting to suppress the color of tobacco and petunia flowers via the transfer of a synthetically created gene designed to turn off (via anti-sense technology) a host pigment gene (van der Krol et al., 1988). The expected outcome was that all the transformed plants would have the same color flowers. However, the transformed plants varied in terms of the amount of color (or pigmentation) in their flowers as well as the pattern of color in the individual flowers. Not only that, but as the season changed (i.e. in different environments), some the flowers also changed their color or color pattern. The factors contributing to the position effect are not fully understood.

The expression of host genes can be influenced by the location of the genetic insertion as well. If the material inserts itself into "the middle" of an important gene, that gene would functionally be turned off. In one experiment, insertion of viral genetic material into a mouse chromosome lead to disruption of a gene which resulted in the death of the mouse embryos (Schnieke et al., 1983). If the "turned off" gene happened to code for a regulatory protein which prevented the expression of some toxin, the net result of the insertion would be to increase the level of that toxin.

The genetic background of the host plant can also affect the level of expression of the transferred gene, which explains the common observation that varieties of the same plant species varied widely in the ease with which they can be genetically engineered (Doerfler et al., 1997; Traavik, 1998). In some varieties, the trait can be expressed at high

enough levels to have the desired impact. In others, the expression level is too low to have the desired impact. In general though, scientists do not really understand why some plant varieties yield more successful results in GE than other varieties.

To get around the common problem of an insufficient level of expression of a desired gene product, powerful regulatory elements—particularly promoters/enhancers—are inserted along with the desired transgene and used to maximize gene expression. The promoter has numerous elements that enable it to respond to signals from other genes and from the environment which tell it when and where to switch on, by how much and for how long. When inserted into another organism as part of a "genetic construct," it may also change the gene expression patterns in the recipient chromosome(s) over long distances up- and downstream from the insertion site. If the promoter (plus associated transgenes) is inserted at very different places on a given chromosome or on different chromosomes, the effects may be very different; it will depend on the nature of the genes that are near the insertion site. This uncertainty of insertion site, along with the promoter means that for all transgenic plants, there will be a fundamental unpredictability with regard to: expression level of the inserted foreign gene(s); expression of a vast number of the recipient organism's own genes; influence of geographical, climate, chemical (i.e. xenobiotics) and ecological changes in the environment; and transfer of foreign genetic sequences within the chromosomes of the host organism, and vertical and/pr horizontal gene transfer to other organisms. Such unpredictability explains the common observations that different insertion events in the same variety can vary greatly in terms of the level of expression of the desired transgene and that the majority of transformation events do not yield useful results (i.e. the transgenic plant is defective in one way or another).

The unpredictable influence of the environment may explain what went wrong in Missouri and Texas with thousands of acres of Monsanto's glyphosate tolerant cotton and Bt cotton, respectively. In Missouri, in the first year of approval, almost 20,000 acres of this cotton in malfunctioned. In some cases the plants dropped their cotton bolls, in others the tolerance genes were not properly expressed, so that the GE plants were killed by the herbicide (Fox, 1997). Monsanto maintained that the malfunctioning was due to "extreme climatic conditions." A number of farmers sued and Monsanto ended up paying millions of dollars in out-of-court settlements. In Texas, a number of farmers had problems with the Bt cotton in the first year of planting. In up to 50% of the acreage, the Bt cotton failed to provide complete control (a so-called "high dose") to the cotton bollworm (*Helicoverpa zea*). In addition, numerous farmers had problems with germination, uneven growth, lower yield and other problems. The problems were widespread enough that the farmers filed a class action against Monsanto. Last fall, Monsanto settled the case out of court, again by paying the farmers a significant sum (Schanks [plaintiffs attorney], personal communication). If there could be this unexpected effect on the growing characteristics of the cotton, it is theoretically possible

that their could be changes in the plant itself which affect the nutritional or safety characteristics of the plant (used as cattle feed) or the seed (the oil from which is used in a number of food products). *This raises the question of whether EPA should establish procedures for assuring safety in the long term.*

The unpredictability associated with the process of genetic engineering itself could lead to unexpected effects such as the production of a toxin that doesn't normally occur in a plant or the increase in a level of a naturally occurring toxin. An example of the former occurred in an experiment with tobacco plants engineered to produce gamma-linolenic acid. Although the plants did produce this compound, another metabolic pathway ended up producing higher quantities of a toxic compound, octadecatetraenic acid, *which does not exist in non-engineered plants* (Reddy and Thomas, 1996).

An example of the latter occurred in an experiment involving yeast where genes from the yeast were duplicated and then reintroduced via genetic engineering (Inose and Murata, 1995). The scientists found that a three-fold increase in an enzyme in the glycolytic pathway, phoshofructokinase, resulted in a 40-fold to 200-fold increase of methylglyoxal (MG), a toxic substance which is know to be mutagenic (i.e. tests positive in an Ames test). This unexpected effect occurred even though the inserted genetic material came from the yeast itself. As the scientists themselves concluded, "Although, except for the case of microbes, we have no information as to the toxic effect of MG in foods on human beings, the results presented here indicate that, in genetically engineered yeast cells, the metabolism is significantly disturbed by the introduced genes or their gene products and the disturbance brings about the accumulation of the unwanted toxic compound MG in cells. Such accumulation of highly reactive MG may cause a damage in DNA, thus suggesting that the scientific concept of "substantially equivalent" for the safety assessment of genetically engineered food is not always applied to genetically engineered microbes, at least in the case of recombinant yeast cells. . . . Thus, the results presented may raise some questions regarding the safety and acceptability of genetically engineered food, and give some credence to the many consumers who are not yet prepared to accept food produced using gene engineering techniques" (Inose and Murata, 1995:   ).

Another study published in *Lancet* in late 1999 used potatoes that were genetically engineered to contain a chemical from the snow drop plant (a lectin, *Galanthus nivalis* agglutinin [GNA]) to increase resistance to insects and nematodes. Feeding experiments with rats demonstrated a number of potentially negative effects (Ewen and Pusztai, 1999). The study found variable effects on the gastrointestinal tract, including proliferation of the gastric mucosa. Interestingly, the potent proliferative effect on the jejunum was seen only in the rats fed GE potatoes with contained the GNA gene but not in rats fed non-transgenic potatoes to which GNA had been added. Indeed, a previous feeding study utilizing GNA with a 1,000-fold higher concentration than the level expressed in the GE potatoes had found no proliferative effect (Pusztai et al.,

1990). The authors proposed "that the unexpected proliferative effect was caused by either the expression of other genes of the construct or by some form of positioning effect in the potato genome caused by GNA gene insertion" (Ewen and Pusztai, 1999: 1354). Such a fine-grained feeding study, which involved utilizing young rats which were still growing and involved weighing various organs and looking very carefully for effects on various organ systems and the immune system is far more detailed than the general feeding studies done utilizing GE plants. While many criticisms have been leveled at this study, we believe it raises important questions that merit further research.

Because of the unexpected effects that are theoretically possible and which have been seen in various experiments, we feel EPA should require long-term animal feeding studies using the whole food product. Such testing should be done on growing animals, so that effects on various organ systems can be readily observed. In addition, fairly extensive data should be taken on the weights of various organs and on histopathology and immunology. In addition, there should be follow-up feeding studies if any data from the lab or field demonstrates that the genetic insert is unstable. FDA should propose its procedures for public comment so that it can get further input from the scientific community and others.

The most commonly used promoter in plant genetic engineering is one from the cauliflower mosaic virus (CaMV); all GE crops on the market contain it. A promoter has numerous elements that enable it to respond to signals from other genes and from the environment which tell it when and where to switch on, by how much and for how long. A CaMV promoter is used for a number of reasons: because it is a very powerful promoter, because it is active in all plants—monocots, dicots, algae—and in *E. coli* and because it is not greatly influenced by environmental conditions or tissue types. CaMV has two promoter, 19S and 35S, but the 35S is the one most frequently used because it is the most powerful. The powerful nature of the CaMV 35S promoter means that it is not readily controlled by the host genes that surround it and often yields a high expression level of the transgene next to it. This is not unexpected as CaMV is a virus that is designed to hijack a plant cell's genetic machinery and make many copies of itself. This also means that it is designed to overcome a plant cell's defensive devices to prevent foreign DNA from being expressed. In the case of transgenic crops, however, the CaMV promoter is used to put the transgenes outside the normal regulatory circuits of the host organism and have them expressed a very high levels. Being placed outside of normal regulatory circuits may be one of the reasons why GEFs are known to be so unstable (Finnegan and McElroy, 1994). The questions raised by the extensive use of the CaMV 35S promoter in engineered crops should be investigated with further research (Ho et al., 1999)

Post-translational processing

Another area of study that raises serious questions about the safety of transgenic traits is the phenomenon of post-translational processing, which consists of the modification of a protein after it has been translated from the genetic message. And such post-translational processing can have a significant impact on the structure and function of a gene. Furthermore, post-translational processing can differ between organisms, so that the same gene expressed in different genetic backgrounds may have the same amino acid sequence but may differ in structure and function. Examples of such processing includes glycosylation, acetylation, and methylation.

Glycosylation and acetylation were covered in the previous section on acute toxicity testing.

Methylation is the process of putting methyl groups on a molecule. Methylation of DNA, which occurs with the nucleotide bases cytosine and adenosine, is important as this appears to prevent that piece of DNA from being expressed (or "turned on"). Methylation is one of the mechanisms behind the phenomenon of "gene silencing," whereby a cell "turns off" a gene. Transgenic work has found that if you try to insert multiple copies of a gene into a plant, the plant will frequently turn off all, or all but one, of the copies of the transgene (Finnegan and McElroy, 1994). Indeed, some scientists now think that gene silencing is an important defense mechanism that plants use to prevent foreign DNA from being expressed (other mechanisms exist to try to degrade the foreign DNA before it can enter the nucleus of the cell) (Traavik, 1998; Ho, 1998). This should be combined with the recent finding that tobacco plants may contain large numbers of copies of paratetroviral-like sequences, in some cases reaching copy numbers of about 10,000 (Jakowitsch et al. 1999). This study is quite striking as it was previously thought that plant viruses rarely integrate, if at all, into host genomes. Furthermore, such integrated viral genetic material is normally silenced via methylation, so that there could be a lot of dormant viral sequences in plants. Interestingly, the cauliflower mosaic virus promoter (CaMV 35) used in virtually all transgenic plants on the market is a pararetrovirus-derived sequence (i.e. CaMV is a pararetrovirus).

With methylation, the danger exists that the CaMV 35S promoter, being a very powerful "on switch" that can have effects thousands of base pairs upstream and downstream from an insertion point, could inadvertently "turn on" a foreign gene that has previously been silent. Given the studies in the last couple of years that suggest that horizontal gene transfer may be more common than previously thought and that most such foreign DNA, if it survives and is able to incorporate itself in the host genome, is frequently "silenced" via methylation, there's a potential risk that some nasty dormant genetic material is inadvertently turned on due to the presence of the CaMV promoter. *Thus, it becomes important to know the exact insertion site of any and all*

genetic construct as well as knowing what the genetic sequence is for thousands of bases pairs upstream and downstream from the insertions site, and do long term toxicity tests with the whole engineered food.

What molecular characterization data EPA should require

Because of all the reasons stated above and because of the random nature of the genetic transformation process each random insertion of transgenic DNA will differ in location and in structure from all other inserts. It will be accompanied by a different pattern of unintended positional and pleiotropic effects due, respectively, to the location of the insert and the functional interaction of the insert with host genes. Thus, each transgenic line resulting from the same process, despite using the same vector system and plant materials under the same conditions will be distinct, and must be treated as such. Consequently, we think EPA should require the companies to submit data for each separate transgenic line. For every line, EPA should require a complete molecular characterization of each line with respect to the identity, stability and unintended positional and pleiotropic effects. And based on the results of such characterization, the agency could decide on how much toxicity data to require.

The components of a complete molecular characterization for molecular identity would include, for each transgenic or transformed line:

- Total number of inserts of transgenic DNA
- Location of each insert (organelle [chloroplast, mitochondria, etc.] or chromosomal)
- Exact chromosomal position of each insert
- Structure of each insert (whether duplicated, deleted, rearranged, etc.)
- Complete genetic map of each insert including all elements (coding region, noncoding regions, marker gene, promoters, enhancers, introns, leader sequences, terminators, T-DNA borders, plasmid sequences, linkers, etc. including any truncated, incomplete sequences)
- Complete (nucleotide) base sequence of each insert
- (Nucleotide) base sequence of at least 10kbp (10,000 base pairs) of flanking host genome DNA on either side of the insert, including changes in methylation patterns

To determine stability, the EPA needs data on both functional stability (level of expression remains constant over time and over successive generations) and structural stability (location in the genome and structural arrangement of the insert). For functional stability, EPA would need data on the level of expression of the transgene over time—throughout the lifetime of the plant as well as over a number of generations (say 3 to 5 generations). For structural stability, the EPA would need data on the physical location of the insert in the genome as well as the structure of the insert—throughout the lifetime of the plant as well as over successive

generations (say 3 to 5). In addition, the EPA should require appropriate molecular probes for each insert with flanking host genome (organelle sequence) sequences in order to monitor the structural stability of the insert.

To test for unintended positional effects, the EPA could look carefully at the methylation patterns of the genes in the flanking host genome DNA (data we suggest be required under molecular identity characterization). To look for pleiotropic (as well as positional effects), each transformed line must be identified in terms of total protein profile and metabolic profiles. The total protein profiles would help to monitor for unintended changes in the pattern of gene expression while the metabolic profile would help to monitor for unintended changes in metabolism. The use of mRNA fingerprinting and protein fingerprinting as part of the protein profiles would represent a better, finer screen for detecting novel biochemical, immunological or toxicological hazards. Some such tests have been suggested by a Dutch government team and should be more carefully considered by the FDA (Kuiper et al., 1998). If any of these tests found differences, there would be more reasons to ask for more comprehensive toxicity testing.

References

Anonymous, 1998. Call for UK genetic food watchdog. *Nature* online service. Sept. 3.

Baur, S. 1998. Characterized allergens causing bakers' asthma. *Allergy*, 53(6): 562-566.

Bernstein, I.L., Bernstein, J.A., Miller, M., Tierzieva, S., Bernstein, D.I., Lummus, Z., Selgrade, M.K., Doerfler, D.L. and V.L. Seligy. 1999. Immune responses in farm workers after exposure to *Bacillus thuringiensis* pesticides. *Environmental Health Perspectives,* 107(7): 575-582

BMA (British Medical Association). 1999. The impact of genetic modification on agriculture, food and health. An interim statement. May, 1999. Board of Science and Education, British Medical Association.

Crippa, M. and G. Pasolini. 1997. Allergic reactions due to glove-lubricant-powder in health-care workers. *International Archives of Occupational Environmental Health,* 70(6): 399-402.

Csordas, A. 1990. On the biological role of histone acetylation. *Biochemistry Journal*, 265: 23-38.

Doerfler, W. et al. 1997. Integration of foreign DNA and its consequences in mammalian systems. *Trends in Biotechnology*, 312: 401-406.

European Commission Services. 2000. Responses to the TACD (Trans Atlantic Consumer Dialogue) Recommendations on Food. Sept. 14, 2000.

Ewen, S.W.B. and A. Pusztai. 1999. Effect of diets containing genetically modified potatoes expressing *Galvanthus nivalis* lectin on rat small intestine. *The Lancet*, 354 (9187): 1353-1354.

Finnegan, H. and McElroy. 1994. Transgene inactivation: plants fight back! *Bio/Technology*, 12: 883-888.

Fox, J.L. 1997. Farmers say Monsanto's engineered cotton drops bolls. *Nature Biotechnology*, 15: 1233.

Harbour G.C., Garlick, R.L., Lyse, S.B., Crow, F.W., Tobins, R.H. and J.G. Hoogerheide. 1992. N-϶-acetylation can occur at lysine residues 157, 167, 171 and 180 of recombinant bovine somatotropin. *Techniques in Protein Chemistry III*: 487-495.

Ho, M.W. 1998. *Genetic Engineering: Dream or Nightmare?* Gateway Books, Bath, United Kingdom.

------------, Ryan, A. and J. Cummins. 1999. The cauliflower mosaic virus promoter—a recipe for disaster? *Microbial Ecology in Health and Disease*, (in press).

Inose, T. and K. Murata. 1995. Enhanced accumulation of toxic compound in yeast cells having high glycolytic activity: A case study on the safety of genetically engineered yeast. *International Journal of Food Science and Technology*, 30: 141-146.

Jakowitsch, J., Mette, M.F., van der Winden, J., Matzke, M.A. and A.J.M. Matzke. 1999. Integrated pararetroviral sequences define a unique class of dispersed repetitive DNA in plants. *Proceedings of the National Academy of Sciences*, 96(23): 13241-13246.

Kuiper, H.A. et al. 1998. *Food Safety Evaluation of Genetically Modified Foods as a Basis for Market Introduction.* Ministry of Economic Affairs, The Hague, Netherlands.

Lorenz, M.G. and W. Wackernagel. 1994. Bacterial gene transfer by natural genetic transformation in the environment. *Microbial Reviews,* 58: 563-602.

Maturin, L. and R. Curtiss. 1977. Degradation of DNA by nucleases in intestinal tract of rats. *Science*, 196: 216-218.

McAllan, A.B. 1982. The fate of nucleic acids in ruminants. *Progress in Nutrional Science*, 41: 309-317.

Mercer, D.K., Scott, K.P., Bruce-Johnson, W.A., Glover, L.A. and H.J. Flint. 1999. Fate of free DNA and transformation of the oral bacterium *Streptococcus gordonii* DL1 by plasmid DNA in human saliva. *Applied and Environmental Microbiology,* 65: 6-10.

Park, H.S., Nahm, D.H., Kim, H.Y., Suh, C.H. and K.S. Kim. 1998. Role of specific IgE, IgG and IgG4 antibodies to corn dust in exposed workers. *Korean Journal of Internal Medicine,* 13(2): 88-94

Reddy, S.A. and T.L. Thomas. 1996. Expression of a cyanobacterial delta 6-desaturase gene results in gamma-linolenic acid production in transgenic plants. *Nature Biotechnology*, 14: 639-642.

Sampson, H.A., L. Mendelson, and J.P. Rosen. 1992. Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *New England J. Med.* 327: 380-384.

Schnieke, Harbers, and Jaenisch. 1983. Embryonic lethal mutation in mice induced by retrovirus insertion into the alpha-1(I) collagen gene. *Nature,* 304: 315-320.

Schubbert, R., Lettmann, C. and W. Doerfler. 1994. Ingested foreign (phage M13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice. *Molecules, Genes and Genetics*, 242: 495-504.

Spring, S. et al. 1992. Phylogenetic diversity and identification of nonculturable mangeto-tactic bacteria. *Systematics and Applied Microbiology*, 15: 116-122.

Steffan, R.J. et al. 1988. Recovery of DNA from soils and sediments. *Applied Environmental Microbiology*, 54: 2908-2915.

Traavik, T. 1998. Too early may be too late: Ecological risks associated with the use of naked DNA as a tool for research, production and therapy. Directorate for Nature Research. Trondheim, Norway.

Van der Krol;. Lenting, and Veenstra et al. 1988. An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. *Nature,* 333: 866-869.

Violand, B.N., Schlittler, M.R., Lawson, C.Q., Kane, J.F., Siegel, N.R., Smith, C.E., Kolodziej, E.W. and K.L. Duffin. 1994. Isolation of *Escherichia coli* synthesized recombinant eukaryotic proteins that contain ε-N-acetyllysine. *Protein Science,* 3: 1089-1097.

Wohlford-Lenane, C.L., Deetz, D.C. and D.A. Schwartz. 1999. Cytokine gene expression after inhalation of corn dust. *American Journal of Physiology,* 276(5 Pt. 1): L736-743.