U.S. Food and Drug Administration

Food

Use of Antibiotic Resistance Marker Genes in Transgenic Plants

September 4, 1998

Guidance for Industry
Use of Antibiotic Resistance Marker Genes in Transgenic Plants
Draft Guidance

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Antibiotic resistance genes are present in transgenic plants as a result of use as marker genes to select transformed plant cells. These genes are under the control of eukaryotic promoters and are expressed in the transgenic plant. Transgenic plants may also contain antibiotic resistance genes that are under the control of prokaryotic promoters, and therefore, not expressed. The latter are incorporated into plant genomes because they are present on constructs used to transform plant cells, having been used for selection in bacteria in earlier steps. In either case, crop developers should evaluate the safety of use of antibiotic resistance marker genes present in transgenic crops.

An evaluation of the safety of use of an antibiotic resistance marker, if it is expressed, should include an assessment of the safety of the protein or enzyme encoded by the gene, if present in food. Regardless of whether it is expressed, crop developers should evaluate the potential for therapy with antibiotics to be compromised through transfer of the gene from plants to microorganisms in the gut of man or animal, or in the environment.

Safety evaluation of a protein encoded by an antibiotic resistance marker gene should include 1) an assessment of potential toxicity of the protein, 2) an assessment of whether the protein has the potential to elicit allergenic reactions, and 3) an assessment of whether the presence in food of the enzyme or protein encoded by the antibiotic resistance marker gene would compromise the therapeutic efficiency of orally administered antibiotic.

FDA acknowledges that the likelihood of transfer of an antibiotic resistance marker from plants to microorganisms in the gut or in the environment is remote and that, such transfer, if any, would likely be insignificant when compared to transfer between microorganisms, and in most cases, would not add to existing levels of resistance in bacterial populations in any meaningful way. Nonetheless, FDA believes that developers should evaluate the use of antibiotic resistance marker genes in crops on a case-by-case basis taking into account information on 1) whether the antibiotic is an important medication, 2) whether it is frequently used, 3) whether it is orally administered, 4) whether it is unique, 5) whether there would be selective pressure for transformation to take place, and 6) the level of resistance to the antibiotic present in bacterial populations. If a careful evaluation of the data and information suggests that the presence of the marker gene or gene product in food or feed could compromise the use of the relevant antibiotic(s), the marke gene or gene product should not be present in the finished food or feed. FDA notes that certain antibiotics are the only drug available to treat certain clinical conditions (e.g., vancomycin for use in treating certain

staphylococcal infections). Marker genes that encode resistance to such antibiotics should not be used in transgenic plants.

The guidance represents the agency's current thinking on the use of antibiotic resistance marker genes in transgenic plants. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. An alternative approach may be used if such an approach satisfies the requirements of the applicable statute, regulations, or both.

Report on Consultations Regarding Use of Antibiotic Resistance Marker Genes in Transgenic Plants

Appendix 1. Evaluation of the Safety of the Kanamycin Resistance Gene as a Selectable Marker

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Consultations Regarding the Use of Antibiotic Resistance Marker Genes in Transgenic Plants

I. Need for Consultations

In 1992, the Food and Drug Administration (FDA) issued a policy statement regarding foods derived from new plant varieties including those derived using genetic engineering techniques (U.S. FDA, 1992). In this policy statement, FDA specifically discussed antibiotic resistance selectable marker genes(1) and noted that both the antibiotic resistance gene and gene product, unless removed, are expected to be present in foods(2) derived from plants developed using the markers. The agency acknowledged that selectable marker gene-encoded enzymes that inactivate certain clinically useful antibiotics, when present in food, theoretically might reduce th therapeutic efficacy of antibiotics administered orally. Thus it is important to evaluate such concerns with respect to commercial use of antibiotic resistance marker genes in food, especially those that will be widely used. In addition, the agency believes it is important to consider the possibility that resistance to antibiotics in microorganisms might spread through potential horizontal transfer of antibiotic resistance marker genes from plants to microorganisms in the gastrointestinal tract or in the environment.

Since FDA's decision regarding the use of the kanamycin resistance (*kan*^{r}) gene product, aminoglycoside 3'-phosphotransferase II (APH(3')II, also known as neomycin phosphotransferase II or nptII) in the developmen of transgenic tomato, cotton, and oilseed rape (U.S. FDA, 1994, see also Appendix 1), the agency has continued to receive inquiries regarding the safety and regulatory status of antibiotic resistance marker genes Therefore, FDA sought to develop sound scientific principles regarding the safety of the use of antibiotic resistance marker genes in the development of transgenic plants for food use and to provide sound scientific guidance to crop developers regarding the safe use of antibiotic resistance marker genes. Towards this end, FDA undertook several consultations with outside experts between November, 1996 and February, 1997. The purpose of the consultations was to determine whether circumstances exist under which FDA should recommend that a given antibiotic resistance gene not be used in crops intended for food use, and if so, to delineate the nature of those circumstances.

In order to facilitate the consultations, the agency developed several questions to form the basis of the discussions with the outside experts. These included: What are the therapeutic uses of the antibiotics that th marker gene products inactivate and how widely are they used? How prevalent is resistance to these antibiotics among bacteria naturally found in the gut or in the environment? For each antibiotic resistance marker gene that is expressed in a transgenic plant, what is the likelihood that clinical therapy could be compromised due to inactivation of the oral dose of the antibiotic from consumption of processed or unprocessed food derived from the transgenic plant? What is the likelihood that events such as rearrangement, recombination, and translocation, would result in changing the expression of the antibiotic resistance marker gene? What is the likelihood that the therapeutic use of an antibiotic could be compromised from transfer of the antibiotic resistance gene from food to gut epithelium with subsequent expression? What is the likelihood that antibiotic resistance marker genes could be transferred from transgenic plants to soil microorganisms with subsequent expression of the gene? How meaningful is the potential rate of transfer, if any, of an antibiotic resistance gene to pathogenic microorganisms thereby rendering them refractory to the antibiotic? Are there any other issues or other information regarding antibiotic resistance marker genes that the agency should take into account in developing guidance to industry on the selection and use of these genes?

II. Format of the Consultations and List of Experts

The experts came to Washington, DC, to engage in separate discussions with a team of scientists from FDA's Center for Food Safety and Applied Nutrition (CFSAN), Center for Veterinary Medicine (CVM), and Center for Drug Evaluation and Research (CDER). Prior to their arrival, the experts were provided with background material including 1) FDA's policy regarding foods derived from new plant varieties, which was published in the May 29, 1992 issue of the *Federal Register,* 2) the final rule authorizing the use of the *kan*^{r} gene product, APH(3')II, in the development of transgenic tomato, cotton and oilseed rape, which was published in the May 23, 1994 issue of the *Federal Register,* 3) the proceedings of a World Health Organization workshop on antibiotic resistance marker genes that took place on September 21-24, 1993, in Copenhagen, Denmark, 4) a document by the UK Advisory Committee on Novel Foods and Processes on the use of antibiotic resistance markers in transgenic plants, 5) a Nordic Council of Ministers document entitled "Health Effects of Marker Gene in Genetically Engineered Food Plants," 6) proceedings of a Joint FAO/WHO Expert Consultation on Biotechnology and Food Safety that was held from September 30 to October 4, 1996, 7) an Environmental Protection Agency final rule that established an exemption from the requirement of a tolerance for residues of

APH(3')II in transgenic plants when used as a plant pesticide inert ingredient, 8) the consultation procedures FDA has been following for foods and feeds derived from transgenic plants, and 9) a list of products on which such consultations have been completed, together with a recent example of a memorandum to the file, and a letter that is issued to the crop developer to signify official closure of the consultation.

Each consultation began with an overview of FDA's food biotechnology policy, the marker genes used in transgenic plants and those that the agency has seen in its consultations to date, and a review of FDA's evaluation of the food safety and environmental safety of *kan*^{r} gene and its gene product, APH(3')II, for use in developing transgenic tomato, cotton, and oilseed rape.

The following experts participated.

- Mitchell L. Cohen, M.D., Director, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA.
- Michael Gilmore, Ph.D., Professor, Department of Medicine, University of Oklahoma, Oklahoma City, OK.
- Donald Lein, D.V.M., Head, Diagnostics Laboratory, Cornell University College of Veterinary Medicine, Ithaca, NY.
- Abigail Salyers, Ph.D., Professor, Dept of Microbiology, University of Illinois, Urbana, IL.
- Kenneth Wilson, M.D., Professor of Medicine, Duke University, Infectious Diseases Section, VA Medical Center, Durham, NC.

III. Summary of the Consultations

A. Direct Effects of Ingestion of Enzymes Encoded by Antibiotic Resistance Marker Genes

Throughout the consultations, the potential effects due to ingestion of enzymes encoded by antibiotic resistance genes as components of food raised little concern in comparison to potential health effects from transfer of antibiotic resistance genes to microorganisms. The potential safety issues, whether toxicity or allergenicity of the gene product, or potential inactivation of an orally administered antibiotic by the gene product, could be addressed by considerations similar to those the agency undertook in its evaluation of the safety of APH(3')II (U.S. FDA, 1994).

For example, digestibility of the protein product by digestive enzymes, functional similarity to other proteins in the diet, lack of sequence homology to known toxins in the sequence databases, and lack of reported toxicity in the literature could be used as indications that the protein does not have toxic properties. In addition, since most of these proteins are likely to be from sources that are not known to be allergenic, *in vitro* or *in vivo* tests for allergenicity would not be useful. However, it was agreed that a determination that the protein does not have any of the properties that are common to allergenic proteins, such as resistance to digestion, heat or acidic pH, coupled with lack of homology to known allergenic proteins in the protein and nucleic acid sequence databases, can give some assurance that a protein encoded by an antibiotic resistance marker gene will not elicit allergenic reactions when consumed as a component of food.

With respect to whether an antibiotic resistance gene product found in food could compromise therapy with a clinically useful antibiotic by inactivating it *in situ*, some assurance against this possibility could be obtained by showing that the gene product is readily digestible or is inactivated by processing methods used by food producers. In addition, if the gene product requires a cofactor in order to inactivate the antibiotic, one could examine whether the concentration of cofactor would be limiting through theoretical calculations. Cofactor requirements could also be addressed through direct experimentation. Finally, in the event of some potential inactivation of antibiotic, advice could be provided that the antibiotic likely to be affected should not be taken together with food. In cases where this is unavoidable and some inactivation is anticipated, increased doses could be given to make up for the fraction of antibiotic that would be inactivated provided that amount can be determined.

In the case of animal feed, it is likely that production methods will denature the enzyme thereby rendering it inactive against the antibiotic. Further, it was noted that most antibiotics are delivered through drinking water rather than feed due to cost considerations. Where antibiotic is added to animal feed, the potential for antibiotic inactivation could be determined by preparing medicated feed from the transgenic plant and analyzing for any loss of potency of antibiotic. This procedure was used by Calgene Inc., for feed containing neomycin prepared from transgenic oilseed rape and cotton containing APH(3')II (U.S. FDA, 1994).

B. Potential Transfer of Antibiotic Resistance Marker Genes to Gut Epithelial Cells

Several issues were considered by FDA in its approval of Calgene's food additive petition for the use of APH(3')II with respect to why potential transfer and expression of the *kan*^{r} gene in gut epithelial cells do not raise a safety concern. Transfer is considered to be unlikely because the DNA is degraded by nucleases and even if some were to survive digestion and were transferred, integrated and expressed, epithelial cells are short lived and would slough off to be replaced by untransformed cells. The above considerations would also be applicable to evaluation of other antibiotic resistance marker genes. Although a large amount of food-derived DNA regularly passes through the gastrointestinal tract, there are no published reports on the

transfer, integration or expression of genes in cells lining the gut. Some experts cautioned that one should assume that DNA can get into the cells lining the gut[3]; however, the critical factor is the lack of selective pressure. Without selective pressure, it is highly unlikely that genes taken by these cells would be expressed even if integrated into the genome. In addition, these cells are sloughed regularly and replaced by new cells. Finally, crypt cells are not sloughed off; however, even if DNA containing an antibiotic resistance gene could ge into these cells and integrate into their genomes, lack of selective pressure makes it unlikely that the gene would be expressed.

C. Potential Transfer of Antibiotic Resistance Marker Genes to Gut Microorganisms

It is highly unlikely that antibiotic resistance genes could be transferred from plant genomes to gut microorganisms. First, there are no known mechanisms for the direct transfer of plant genomic DNA to microorganisms. Second, there are several barriers to potential transfer. These include degradation by acid and nucleases in the stomach and intestines, the bacterial restriction and modification systems that destroy foreign DNA that enters the cell, the absence of homologous ends for efficient integration into the bacterial genome, and lack of selective pressure.

In addition, when any DNA (including antibiotic resistance genes) is integrated into plant genomes, the codon usage may have been altered for more efficient expression in the plant and the gene may have picked up methylation patterns of the plant. If this DNA is now taken up by a bacterium, it would be recognized as foreign and degraded by the microorganism's restriction endonucleases, thus making integration into the genome and subsequent expression even more unlikely. Moreover, transfer between bacteria, even among broadly different phylogenetic lines, is far more likely than transfer from plants to bacteria. Finally, since uptak is usually not sequence-specific, the antibiotic resistance gene would be competing for transfer into a bacterium with the rest of the DNA in the plant genome and DNA from other sources in the diet[4].

Nonetheless, the possibility was raised that unlikely events could take place given sufficient selective pressure and that, because of the short generation times of bacteria, clonal expansion of the transformed bacteria coul take place. For these reasons, and because some antibiotics are so important clinically, it is prudent for developers to ensure that marker genes that encode resistance to clinically important antibiotics are not present in food or feed derived from new plant varieties. For example, vancomycin was cited as a drug of last resort for some staphylococcal and enterococcal infections. Additional critical antibiotics mentioned by some of the experts were other glycopeptides, fluoroquinolones, tetracycline, gentamicin and the later derivatives of *beta*-lactam antibiotics[5].

Overall, the arguments made concerning the improbability of the transfer of the *kan*^{r} gene to gut microorganisms in the case of the Flavr Savr™ tomatoes (U.S. FDA, 1994) could also be applied to other antibiotic resistance marker genes. However, while the possibility of transfer from plants to microorganisms is remote for all marker genes, crop developers should consider the following factors in evaluating whether an antibiotic resistant gene is suitable for use as a selectable marker: 1) whether the antibiotic that may be affected is clinically important, 2) whether it is frequently used, 3) whether it is administered orally, 4) whether it is unique or acceptable alternative antibiotics exist, 5) whether there would be selective pressure for transformants to be selected, and 6) whether there already is resistance to the antibiotic in the environment.

FDA's analysis (U.S. FDA, 1994) showed that the *kan*^{r} gene that was used in the case of the Flavr Savr™ tomato passes the paradigm outlined above. Neomycin and kanamycin are infrequently used antibiotics, neither is unique for any use, and rarely are administered orally. Thus, selective pressure would be minimal fo development of resistant bacteria because the drugs are not used in humans or in animals to any great extent. Similarly, these antibiotics are not used in agriculture or aquaculture to any great extent. Therefore, they would not provide the selective pressure required to select for the *kan*^{r} determinant if transfer from plant to soil microorganisms were to take place. In addition, existing resistance levels far exceed any transfer that may take place from transgenic plants to microorganisms.

However, different circumstances may apply to other antibiotics. For example, with regard to the presence or absence of selective pressure, streptomycin and oxytetracycline may provide selective pressure in the environment because of their use as pesticides in agriculture. On the other hand, ampicillin may provide selective pressure in the human gut when used in the clinical setting but not in the environment.

The notion that it may be possible to construct a list of antibiotic resistance marker genes that are acceptable for use in the development of transgenic crops was discussed. The *kan*^{r} gene can be placed on such a list. Some experts suggested that the hygromycin resistance gene may be included on such a list because of its limited use in humans. It was noted, however, that it may have important veterinary uses. Other experts would include the *beta*-lactamase gene of pUC18 (that confers resistance to a narrow spectrum of *beta*-lactam antibiotics), and the tetracycline resistance gene on such a list. There is so much resistance to these antibiotic already in the environment that any potential transfer from transgenic plants to microorganisms, especially when compared to transfer among bacteria, is unlikely to add to the existing levels of resistance in any

meaningful way.

The idea was put forth that antibiotic resistance genes could be ranked on a continuum with the *kan*^{r} gene on one end as the most acceptable, and the vancomycin resistance gene on the other. It was further suggested that use of marker genes beyond the *kan*^{r} and hygromycin resistance genes might be acceptable on the basis of studies to address potential transfer and a commitment to conduct post-market surveillance for transfer of the gene in question (see below).

D. Potential Transfer of Antibiotic Resistance Marker Genes to Microorganisms in the Environment

Some experts noted that some soil microbes may be naturally transformable and that they may take up and incorporate DNA causing genomic rearrangements that might help them occupy particular ecological niches. Some experts felt that it does not make sense to expand the availability of the resistance gene in the environment because abundance of marker genes may compress the typical 4- 5-year time lag between first use of new antibiotic and the emergence of resistance in hospitals. Others felt that the risk of transfer from plant genome to soil microorganisms is not a significant one. This latter group felt that DNA from plant debris would be unavailable for transfer because it would be degraded by nucleases when the plant cell lysed; in addition, there would be no selective pressure in most cases although there are exceptions such as when streptomycin and tetracycline are used as pesticides to prevent fire blight in fruit trees, or when manure is used as fertilizer following use of antibiotics as growth promoters in animals. It was noted that transfer from bacteria to bacteria accounts for the wide dissemination of certain antibiotic resistance markers in soil bacteria

E. Approaches to Assessing Potential for Transfer of Antibiotic Resistance Marker Genes and Conducting Surveillance for Resistance

Participants in the consultations discussed a study conducted by a crop developer wherein plant DNA containing the *beta*-lactamase gene from an insect-resistant transgenic corn line (intact or nuclease degraded) was incubated with competent *E. coli*. The study was intended to determine if any bacteria were transformed and acquired ampicillin resistance; the experiment showed that transformation did not occur above a frequency of 1 in 6.8 x 10^{19}. Some experts said that if transformation were to take place, it would be more likely to do so in experiments using competent bacteria in the laboratory than in nature because competent bacteria have the highest transformation frequency. They added that if transformation was not observed in the laboratory, especially if the experiment was carried out in gram negative and gram positive bacteria (an *Enterococcus* strain and an *E. coli*), the results would suggest that such transfers may not take place in the natural setting to the extent that they would raise health or safety concerns.

Other experts stated that an *in vitro* experiment does not give them much confidence because it does not reflect the complex ecological system that exists in nature. In addition, a monoculture of *E. coli*, is an artificial system that would not be a strong basis on which to assess risk.

The types of experiments that are conducted should be commensurate with the importance of the antibiotic that may be compromised. While an *in vitro* model would suffice for an antibiotic that is relatively unimportant clinically, studies in animals may be warranted for important antibiotics. Some experts suggested that the conclusion could be made that transfer does not take place if a large number of animals were fed bioengineered plants containing an antibiotic resistance gene under intense selective pressure, and new resistant microorganisms with this genotype were not observed.

Some experts also suggested that FDA might consider requiring crop developers to monitor for the spread of resistance due to use of an antibiotic resistance marker in a transgenic plant, especially if the gene confers resistance to a clinically important antibiotic. However, it would be a difficult task to document transfer due to the high levels of resistance that already exist. Monitoring, if conducted, should be at the genetic level rather than at the phenotypic level and, given that all resistance genes originate from microorganisms, it should distinguish a gene that has been transferred from a plant (for example, by looking for unique sequences that may be embedded in the gene in question). It was noted that examining areas with a high concentration of transgenic biomass would increase the chances of finding the rare transfer event. Alternatively, monitoring antibiotic resistance markers where the antibiotic is used in feed as a growth promoter would increase the chance of finding a microbial transformant because there would be selective pressure.

Because the argument is often made that the rate of transfer of an antibiotic resistance marker gene to microorganisms in the gut would be so low as to be meaningless when compared to existing levels of antibiotic resistance, some experts suggested that a survey should be undertaken of present levels of antibiotic resistance among several microbial populations. While such data exist for some resistance genes and for some microorganisms, it was noted that there are gaps in the database. Such a survey would involve isolating DNA from about 100 representatives from each major genus (*Bacteroides, Eubacterium, Bifidobacterium, Peptostreptococcus*, *etc.*). Many of these organisms can be obtained from various laboratories and institutes, and probed for antibiotic resistance marker genes. To look for those genes that occur less commonly, PCR analysis of rectal swabs from humans and animals could be done. Data obtained from these studies would provide evidence concerning the presence of a specific antibiotic resistance gene, and if present, how prevalent it is in the general population of naturally occurring intestinal bacteria.

IV. Conclusions

- The approach taken by FDA in its evaluation of the safety of the use of the *kan*^{r} gene and its product, APH(3')II, in the development of transgenic tomato, cotton, and oilseed rape, is scientifically sound and included all relevant parameters. These included: 1) evaluation of the safety of the protein with respect to toxicity and allergenicity, 2) an assessment of whether presence in food of APH(3')II would compromise the therapeutic efficiency of orally administered neomycin and kanamycin, and 3) an assessment of whether therapy with antibiotics might be compromised through transfer of the *kan*^{r} gene from plants to microorganisms in the gut or in the environment or to the cells lining the gastrointestinal tract.
- The presence in food of proteins encoded by antibiotic resistance genes is not of great concern. They can be evaluated with respect to toxicity and allergenicity and with respect to potential to compromise therapy with antibiotics (in similar fashion to the approach taken by FDA for APH(3')II).
- Similarly, the potential transfer of antibiotic resistance genes from foods derived from transgenic plants to cells lining the gastrointestinal tract does not raise a safety concern. Most DNA is degraded in the gut and thus, would be unavailable for transfer, and even if some DNA survived and was available for transfer into these cells, it would not be integrated and expressed due to lack of selective pressure. Additionally, because these cells are continuously sloughed off and replaced by new cells, a cell that incorporated an antibiotic resistance gene would not be long-lived and present a safety hazard with respect to compromising therapy with antibiotics.
- The likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of man or animal, and in the environment is remote. Several barriers operate against such transfer. In addition, the rate of such transfer, if any, would be insignificant when compared to transfer between microorganisms, and would not add to existing levels of resistance in bacterial populations in any meaningful way. Nonetheless, caution should be the rule for antibiotic resistance markers that inactivate clinically important antibiotics.
- The *kan*^{r} gene is safe to use as a selectable marker in the development of transgenic crops. Some experts also felt that there would be little concern with use of the hygromycin resistance gene as a selectable marker. However, hygromycin may have important veterinary uses and, therefore, its use should be carefully evaluated in those crops that have animal feed applications.
- There are varying levels of concern with use of other antibiotic resistance genes as selectable markers in transgenic plants, with the highest level of concern for those genes that confer resistance to antibiotics such as vancomycin, an antibiotic viewed as a drug of last resort for some infections. Overall, use of these antibiotic resistance marker genes should be evaluated on a case-by-case basis with the evaluation taking into account information on 1) whether the antibiotic is an important medication, 2) whether it is frequently used, 3) whether it is orally administered, 4) whether it is unique, 5) whether there would be selective pressure for transformation to take place, and 6) whether there already is resistance to the antibiotic in bacterial populations.
- Some experts suggested that surveying the current levels of resistance to various antibiotics would be important in order to gauge the impact of any potential transfer of antibiotic resistance genes from transgenic plants to microorganisms. In addition, some experts recommended that developers of transgenic crops might be encouraged to conduct surveillance to ascertain that transfer of antibiotic resistance genes from transgenic plants to microorganisms is not taking place. Such surveillance would safeguard against the occurrence of drug resistance from use of marker genes in transgenic plants and provide the public health community and the regulatory agencies an opportunity for early intervention to prevent adverse impact on public health.

V. References

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4. U.S. Food and Drug Administration, "Secondary Food Additives Permitted in Food for Human Consumption; Food Additives Permitted in Feed and Drinking Water of Animals; Aminoglycoside 3'-Phosphotransferase II; Final Rule," *Federal Register*, 59:26700-26711, 1994.

VI. Notes

(1) Transformation of plant cells by introducing exogenous genes is an inefficient process and only a small proportion of cells successfully take up, integrate, and express the new genetic material. Because the few cells

that do so are not readily distinguishable from the vast majority that do not, developers frequently link an antibiotic resistance marker gene to the gene(s) of interest to allow them to distinguish between transformed and nontransformed cells. Plant cells that are not transformed die when grown in medium containing the antibiotic while those that are transformed live because incorporation and expression of the antibiotic resistance marker gene enables them to counter the action of the antibiotic. Those cells, which contain the marker gene and other gene(s) of interest are subsequently regenerated into transgenic plants.

(2) In this report, the term "food" encompasses both human food and animal feed.

(3) A recent paper (Schubbert, et al., 1997) demonstrated, using phage DNA, that DNA ingested in food is not completely degraded in the gastrointestinal tract of mice, can reach other organs via the intestinal wall mucosa, and can be covalently linked to mouse DNA in these organs.

(4) The fact that an antibiotic resistance gene is under a eukaryotic promoter in the plant is frequently cited as a barrier because, should transfer take place, the eukaryotic promoter would not be able to direct its expression in the microorganism. However, the experts noted that rearrangements, especially under selective pressure, can easily bring a prokaryotic promoter in front of the gene leading to expression.

(5) The pUC19 *beta*-lactamase gene typically seen in recombinant plant genomes under the control of a bacterial promoter would not pose a health hazard should it be transferred and expressed. Unlike the *beta*-lactamase genes that confer resistance not only to a wide variety of *beta*-lactam antibiotics but also to *beta*-lactamase inhibitors that have been used to "recycle" antibiotics (*e.g.* ampicillin) and are currently causing problems in hospitals, this *beta*-lactamase poses no clinical problems because there are many antibiotic formulations that easily control bacterial strains producing it (Salyers, 1996).

Appendix 1. Evaluation of the Safety of the Kanamycin Resistance Gene as a Selectable Marker

I. Background

The kanamycin resistance (*kan*^{r}) marker gene is one of the most widely used selectable marker genes. The *kan*^{r} gene, which was originally isolated as a component of transposon Tn5 from the bacterium *Escherichia coli* (Beck et al., 1982) encodes aminoglycoside 3'-phosphotransferase II (APH(3')II)(6). APH(3')II is an enzyme with an apparent molecular weight of 25,000 that catalyzes the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group of aminoglycoside antibiotics including neomycin, kanamycin, paromomycin, ribostamycin, gentamicins A and B, as well as butirosins, thereby inactivating the antibiotics (Davies et al., 1978; Goldman et al., 1976). Of the antibiotics that are inactivated by APH(3')II, only neomycin and kanamycin are currently in therapeutic use for humans and animals in the United States (U.S. Pharmacopeia, 1990; Prescott et al., 1988)(7).

On November 26, 1990, Calgene, Inc., of Davis, California, submitted to FDA, a request for advisory opinion regarding whether the *kan*^{r} gene may be used as a selectable marker in the production of genetically engineered tomato, cotton, and oilseed rape plants intended for human food and animal feed uses (*kan*^{r} Gene Safety and use in the production of genetically engineered plants, Docket Number 90A-0416). In the May 1, 1991 issue of the *Federal Register*, FDA announced that the request had been received and solicited comments from interested persons (U.S. FDA, 1991). The data submitted to the agency with the request for advisory opinion and the comments received were made available to the public at the Dockets Management Branch.

Subsequently, in January 1993, Calgene requested that FDA convert its request for advisory opinion to a food additive petition under section 409 of the Act. The FDA then announced in the *Federal Register* of July 16, 1993 that a food additive petition had been filed by Calgene, proposing that the food additive regulations be amended to provide for the safe use of APH(3')II as a processing aid in the development of new varieties of tomato, oilseed rape, and cotton (U.S. FDA, 1993).

Calgene had also sought an advisory opinion from the agency on its Flavr Savr™ tomato in a letter dated August 12, 1991. After it completed its evaluation of the safety of the Flavr Savr™ tomato and the use of APH(3')II in the development of tomato, oilseed rape and cotton, FDA, in May, 1994, convened its Food Advisory Committee to deliberate on its evaluation of Calgene's Flavr Savr™. Because the *kan*^{r} gene is the selectable marker used in the Flavr Savr™, the Food Advisory Committee also deliberated on FDA's evaluation of the safety of the use of the selectable marker in the tomato. Following these deliberations FDA amended the food additive regulations to permit the use of APH(3')II in the development of genetically modified tomatoes, oilseed rape, and cotton intended for food use (U.S. FDA, 1994).

Only the product of the *kan*^{r} gene, APH(3')II, and not the gene itself, was regulated as a food additive. The DNA that makes up the *kan*^{r} gene does not differ from any other DNA and does not itself pose a safety concern as a component of food. As the 1992 policy statement made clear, because DNA is present in the cells of all living organisms, including every plant and animal used for food by humans or animals, and is efficiently digested, FDA does not anticipate that DNA would itself be regulated as a food additive. However, as

discussed below, because of the property of the *kan*^{r} gene to confer antibiotic resistance, the agency considered the possibility that the gene might be transferred to other organisms.

II. Issues Considered by The FDA

Safety issues associated with use of APH(3')II in the development of transgenic tomato, cotton and oilseed rape that the agency considered can be divided into two areas: (1) Those associated with the direct effects of ingestion of the protein, including the possibility of allergenicity; and (2) those associated with the biological activity of APH(3')II (*i.e.*, the effect of the enzyme on the therapeutic efficacy of orally administered antibiotics) The agency also evaluated whether there are any safety concerns from potential horizontal transfer of the *kan*^{r} gene in tomato, oilseed rape, and cotton.

A. Direct Effects of Ingestion

The FDA evaluated data that showed that, unlike most allergenic proteins, APH(3')II is rapidly inactivated by stomach acid, is degraded by digestive enzymes (Fuchs et al., 1993), and is not glycosylated when produced i the transgenic tomato, oilseed rape, and cotton. In addition, enzymes such as APH(3')II are heat labile. Thus, APH(3')II does not possess any of the characteristics associated with allergenic proteins such as proteolytic stability, glycosylation, or heat stability (Taylor et al., 1987). In addition, protein and DNA sequence comparisons using sequences in four separate databases (GenBank, EMBL, PIR 29, and Swiss-Prot) showed that APH(3')II does not have significant homology to any proteins listed as food allergens or toxins in these databases.

The agency also noted that it was not aware of any reports that indicate that APH(3')II might be toxic and further noted that all plants and animals that are part of the food supply contain phosphorylating enzymes such as APH(3')II that have been consumed without adverse consequences. Further, acute mouse feeding studies showed that feeding extremely high doses of purified APH(3')II caused no deleterious effects (Fuchs e al., 1993). Finally, FDA calculated that the estimated dietary exposure (EDI) to APH(3')II is very low (480 µg APH(3')II per person per day[(8)], or 0.16 part per million in the diet, based on a 100-percent market share for tomatoes containing APH(3')II). For these reasons, the agency concluded that there are no allergenicity or toxicity concerns from ingestion of the enzyme.

B. Effects on the Therapeutic Efficacy of Orally Administered ntibiotics

1. APH(3')II in human foods

In assessing whether there could be effects on the therapeutic efficacy of orally administered antibiotics, FDA considered only APH(3')II from fresh tomatoes to be relevant because it is the only form that is enzymatically active. Processed tomato products are subjected to high temperatures that would be expected to inactivate the APH(3')II enzyme. For edible oils extracted from cottonseed and rape seed, high temperature treatment, solvent extraction, and subsequent purification steps generally included in the processing of such oils would also be expected to inactivate APH(3')II.

The FDA evaluated several studies intended to address whether APH(3')II consumed as a component of fresh tomatoes could render orally-administered kanamycin ineffective. These experiments were performed under simulated gastric and intestinal conditions (*i.e.*, appropriate pH, reagent concentrations, temperature, and reaction times) chosen to reflect conditions expected *in vivo*. In some studies both tomato extract and nonfat milk were added to determine whether the presence of additional food-source proteins in the simulated gastri and intestinal fluids might slow the proteolytic degradation of APH(3')II by competition. After evaluating data on the loss of immunologically detectable APH(3')II, FDA concluded that, under normal gastric and intestinal conditions, APH(3')II would be effectively degraded before the enzyme could inactivate kanamycin or neomycir and therefore, APH(3')II would not interfere with orally administered kanamycin or neomycin therapy.

In addition, FDA evaluated the results of *in vitro* degradation studies performed under simulated abnormal gastric conditions, such as may exist in patients treated with drugs that reduce stomach acidity. These studies showed that APH(3')II is not degraded in neutralized (pH 7.0) simulated gastric fluid and thus, APH(3')II may remain active in such abnormal gastric conditions. However, FDA concluded that, even under those conditions, APH(3')II would not be expected to inactivate significant amounts of orally administered kanamycin or neomycin because the concentration of ATP, which the enzyme requires to inactivate kanamycin and neomycin would be limiting. The FDA evaluated data from the published literature on ATP levels in fresh fruits and vegetables, estimated ATP intake, and calculated the fraction of neomycin that would be phosphorylated even making the conservative assumption that all of the ATP will survive the intestinal phosphatases and be available to react with the antibiotic. Even under the worst-case situation (high intake of ATP-containing food, low dose of antibiotic) FDA determined that only a small fraction (no more than 1.5 percent) of the antibiotic would be inactivated. This conclusion was supported by data from an *in vitro* study that showed that no significant inactivation of kanamycin was observed when tomato extract containing APH(3')II and kanamycin was incubated over a 4-hour period.

In addition, the agency also considered the patient population likely to be exposed to aminoglycoside antibiotics. Oral aminoglycosides are most commonly administered to either pre-operative patients (prior to bowel surgery) or patients with hepatic encephalopathy. Neither patient population would be expected to be ingesting tomatoes or any other fresh fruits and vegetables; therefore there is little or no risk of inactivating the oral antibiotic in these patients. For these reasons, FDA concludes that the presence of APH(3')II in food will not compromise the therapeutic use of orally administered kanamycin or neomycin.

2. APH(3')II in animal feed

The FDA also considered the potential inactivation of neomycin that is used in animal feeds manufactured usin cottonseed meal and rape seed meal obtained from transgenic plants. The transgenic tomato was not considered because very little tomato and tomato byproducts are used in the animal feed industry. Further, neomycin is primarily used to treat calves and swine whereas tomato byproducts, to the extent that they are used in animal feed, are primarily used as ingredients in cattle diets.

The agency reviewed data on neomycin levels both in nontransgenic medicated cottonseed and rape seed meals and in transgenic medicated cottonseed and rape seed meals over a storage period of 56 days (considered a worst-case situation) and concluded that there was no significant inactivation of neomycin and thus, the therapeutic efficacy of neomycin in animal feed will not be affected. The agency also considers this conclusion applicable to other aminoglycoside antibiotics inactivated by APH(3')II, when orally administered.

C. Potential Transfer of the Kanamycin Resistance Gene

The agency also evaluated issues relevant specifically to the safety of the use of the *kan*^{r} gene in tomato, oilseed rape, and cotton. In particular, FDA evaluated the potential for horizontal transfer of the gene and subsequent expansion of the population of antibiotic-resistant pathogens. The agency evaluated whether efficacy of oral antibiotic treatment of humans or animals could be compromised by consumption of food containing the *kan*^{r} gene either because of transfer of the gene from food to resistant intestinal microflora or to cells lining the intestinal lumen. In addition, the agency considered the possible transfer of the *kan*^{r} gene from transgenic plants to soil microorganisms and expansion of the antibiotic-resistant bacterial population.

1. Potential transfer of the *kan*^{r} gene to intestinal microorganisms and cells lining the intestinal lumen.

The agency evaluated theoretical and experimental evidence that demonstrate that the potential for compromise of antibiotic therapy by horizontal transfer of the *kan*^{r} gene to gut microorganisms or intestinal epithelial cells is not of significant concern. The agency considered potential transfer of the *kan*^{r} gene only fron fresh tomatoes because processing is expected to inactivate the *kan*^{r} gene in processed tomato products and in food and feed products derived from cotton and oilseed rape.

The agency also evaluated *in vitro* data that showed that only 0.1 percent of DNA could be detected as fragments of 1,000 base pairs or longer after exposure to simulated stomach fluids for 10 minutes and to simulated intestinal fluids for another 10 minutes. Thus most of the DNA remaining after digestion would be smaller than the *kan*^{r} gene which is about 1,000 base pairs long and would be unavailable for potential transformation of gut microorganisms. In addition, in animals, even if DNA was not completely degraded by processing during feed production, any remaining DNA would be degraded by the digestive processes. For example, studies have shown that nucleic acids introduced into the rumens of calves, or incubated with calf, sheep, or cow rumen contents *in vitro*, were rapidly and completely degraded to nucleotides and nucleosides (McAllan et al., 1973). Similar results were obtained when DNA was infused into the duodenum of steers (McAllan, 1980). Moreover, many rumen bacterial strains have nuclease activity, which degrades DNA and provides yet another barrier to transformation (Flint and Thompson, 1990).

Finally, Calgene calculated and FDA agreed that, even using worst-case assumptions such as all microorganisms in the intestine being transformation competent, in a person consuming fresh tomatoes at the 90th percentile level, the transformation frequency of intestinal microorganisms with the kan^r gene will be approximately $3x10^{-15}$ transformants per day. This transformation frequency is more than 5 orders of magnitude less than the frequency of mutation to kanamycin resistance per bacterial replication, *i.e.*, 10^{-9} (Davies, 1986). Thus, for every 300,000 bacteria that mutate to kanamycin resistance per replication (generall a matter of hours), there would be, at most, under worst-case conditions, one kanamycin-resistant bacterium per day added to that number due to transformation.

The potential for food-producing animals to experience decreased efficacy of antibiotic therapy as a result of pathogenic intestinal microflora incorporating and expressing the *kan*^{r} gene would be similar to that described for humans, *i.e.*, equally improbable because the worst-case transformation scenario described above for human gut microorganisms also applies to microorganisms found in the gut of food-producing animals.

With respect to epithelial cells lining the intestinal lumen, no transformation of human epithelial cells has been demonstrated *in vivo* (Hoskins, 1978). In addition, even if transformed, intestinal epithelial cells are terminally differentiated (*i.e.*, do not divide) and have a relatively short life span, and thus would continually be shed and

replaced by nontransformed cells.

2. Potential transfer of the kanamycin resistance gene to soil microorganisms

The FDA also considered the possibility that the *kan*^{r} gene might be transferred to soil microorganisms, thereb increasing the level of antibiotic-resistant organisms in the environment. A major barrier to transformation is expected to be the rapid degradation of plant DNA by plant nucleases that takes place when the cell breaks up. Similar calculations to the above showed that, under worst-case assumptions, kanamycin-resistant transformants resulting from plant DNA left in the fields would represent not more than one in 10 million of the existing kanamycin-resistant soil population. Biosafety of use of the *kan*^{r} marker gene in transgenic plants has also been discussed elsewhere (Nap et al., 1992, Redenbaugh et al., 1994).

D. Food Advisory Committee Discussions Regarding Potential Horizontal Transfer of the *kan*^{r} Gene

The FDA's Food Advisory Committee has discussed the possibility that the *kan*^{r} gene might be transferred to microorganisms in the GI tract and in the environment. The committee members concluded that transfer of the *kan*^{r} gene consumed as a component of tomatoes to microorganisms in the GI tract was highly unlikely based on published data in the scientific literature. Similarly, the committee members judged that the potential for transfer of the *kan*^{r} gene from plants to microorganisms in the environment is highly unlikely based on current knowledge of mechanisms of gene transfer. In addition, members of the committee pointed out that the rate at which such transfer could take place, if at all, was of so small a magnitude that, coupled with the high prevalence of kanamycin resistant organisms already present in the environment, it would not cause a significant environmental impact.

Some members of the committee, while convinced by the information presented at the meeting that the transfer of the *kan*^{r} gene from tomato plants to microorganisms in the soil was improbable, expressed concern regarding the use of the *kan*^{r} gene in other crops that may be grown on a wide scale. In addition, some committee members were concerned that a determination of safety with regard to the use of *kan*^{r} gene in Calgene's tomato might signal to producers that it is now permissible to use the *kan*^{r} gene in other crops. In light of such concerns, these committee members advised that use of the *kan*^{r} gene in other crops should be evaluated on a case-by-case basis.

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IV. Notes

(6) Other names for this enzyme include neomycin phosphotransferase II (NPT II), neomycin phosphotransferase and kanamycin phosphotransferase II.

(7) Gentamicin, which is used for human therapeutic use, is composed of a complex mixture of the antibiotic substances produced by *Micromonospora purpurea* and contains primarily gentamicin C_1 (25-50%), gentamicin C_{1a} (10-35%), and gentamicins C_{2a} and C_2 (25-55%) (9). Gentamicins A and B are at most minor components of the commercial drug. Thus, APH(3')II does not confer resistance to gentamicin that is used therapeutically (Davies, 1986).

(8) Because oils produced from transgenic cottonseed and rape seed would not contribute APH(3')II to the human diet, the exposure estimate was derived exclusively for tomatoes. The agency made several conservative assumptions in arriving at its EDI for APH(3')II of 480 µg/person/day. For example, FDA assumed that all tomatoes contain APH(3')II at a level of 0.1 percent of total protein although, of the two lines intended for commercialization by Calgene, one contains less than 0.01 percent and the other less than 0.002 percent of APH(3')II (as a percentage of total protein). Second, FDA included APH(3')II in processed products in its estimate although high temperature treatment used in the production of processed tomato products would be expected to result in loss of enzymatic activity of APH(3')II.

Appendix 2. Review of FDA/Industry Consultations Regarding New Plant Varieties and Selectable Markers Used to Develop Them

I. Purpose of the Consultations

Following evaluation and approval of the Flavr Savr™ tomato in 1994, FDA has not found it necessary to conduct comprehensive scientific reviews of foods derived from bioengineered plants based on the attributes of these products, but consistent with its 1992 policy, FDA expects developers to consult with the agency on safety and regulatory questions. Such consultations will facilitate resolution of safety and regulatory issues. Many firms have initiated consultations with the agency early in the research and development phase of their product.

At some stage in the process of research and development, a firm will have accumulated the information that i believes is adequate to ensure that the product is safe and complies with the relevant provisions of the Act. The firm will then be in a position to conclude any ongoing consultation with FDA. To inform FDA about bioengineered foods that are intended for commercial distribution, the agency recommends that the crop developer submit to FDA, a summary of the safety and nutritional assessment that has been conducted, and if necessary, meet with agency scientists to discuss the scientific data and information that support the summar of the safety and nutritional assessment.

The safety and nutritional assessment summary typically contains sufficient information for agency scientists to understand the approach the firm has followed in identifying and addressing relevant issues and includes,

1. The name of the bioengineered food and the crop from which it is derived.
2. A description of the various applications or uses of the bioengineered food, including animal feed uses.
3. Information concerning the sources, identities, and functions of introduced genetic material.
4. Information on the purpose or intended technical effect of the modification, and its expected effect on th composition or characteristic properties of the food or feed.
5. Information concerning the identity and function of expression products encoded by the introduced genetic material, including an estimate of the concentration of any expression product in the bioengineered crop or food derived thereof .
6. Information regarding any known or suspected allergenicity and toxicity of expression products and the basis for concluding that foods containing the expression products can be safely consumed.
7. Information comparing the composition or characteristics of the bioengineered food to that of food derived from the parental variety or other commonly consumed varieties with special emphasis on important nutrients, and toxicants that occur naturally in the food.

8. A discussion of the available information that addresses whether the potential for the bioengineered food to induce an allergic response has been altered by the genetic modification.
9. Any other information relevant to the safety and nutritional assessment of the bioengineered food.

The goal of FDA's evaluation of information on new plant varieties provided by developers during the consultation process is to ensure that human food and animal feed safety issues or other regulatory issues (*e.g.* labeling) are resolved prior to commercial distribution. During the consultation process, FDA does not conduct a comprehensive scientific review of data generated by the developer. Instead, FDA considers, based on agency scientists' evaluation of the available information, whether any unresolved issues exist regarding the food derived from the new plant variety that would necessitate legal action by the agency if the product were introduced into commerce. Examples of unresolved issues may include, but are not limited to, significantl increased levels of plant toxicants or anti-nutrients, reduction of important nutrients, new allergens, or the presence in the food of an unapproved food additive. The FDA considers a consultation to be completed when all safety and regulatory issues are resolved.

In 1994, FDA discussed this consultation process during a public joint meeting of the agency's Food and Veterinary Medicine Advisory Committees, which consist of food and feed safety experts from outside the agency. At this meeting, FDA summarized safety and nutritional information provided by developers on seven genetically engineered foods: three improved softening or ripening tomatoes, a virus-resistant squash, an insect-resistant potato, and herbicide-tolerant cotton and soybean. The committee members agreed with FDA that, based on the types of bioengineered foods and feeds under development, the consultation procedures provide an appropriate level of government oversight.

II. Bioengineered Foods on Which Consultations Have Been Completed

In addition to the Flavr Savr™ tomato, the agency has completed thirty consultations; seven each in 1994 an 1995, ten in 1996, and six in 1997. These consultations are listed below and are categorized by crop. The trai of the new variety, the gene responsible for the trait, and the source organism of the gene are also given. In addition, the firms that undertook the consultations with FDA and the year in which the consultations were completed are given in parenthesis. A list of consultations that are completed as well as the consultation procedures can be accessed at FDA's home page on the World Wide Web at HTTP://WWW.FDA.GOV under Center for Food Safety and Applied Nutrition. Note that the listed products may have pending regulatory issue with EPA or USDA/APHIS.

A. New Corn Varieties

1. Glufosinate Tolerant
    a. Phosphinothricin acetyltransferase gene from *Streptomyces viridochromogenes* (AgrEvo, 1995)
    b. Phosphinothricin acetyl transferase gene from *Streptomyces hygroscopicus* (Dekalb Genetics, 1996)
2. Male Sterile
    a. Barnase gene from *Bacillus amyloliquefaciens* (Plant Genetics System, 1996)
3. Insect-Protected
    a. Cry1A(b) from *Bacillus thuringiensis* subsp. *kurstaki* (two consultations by Monsanto, 1996; Northrup King, 1996; Ciba-Geigy, 1995)
    b. CryIA(c) gene from *Bacillus thuringiensis* (Dekalb Genetics, 1997)
4. Insect-Protected and/or Glyphosate-Tolerant
    a. CryIA(b) from *Bacillus thuringiensis* subsp. *kurstaki* and/or enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (Monsanto, 1996

B. New Tomato Varieties

1. Modified Fruit Ripening
    a. S-adenosylmethionine hydrolase from *E. coli* bacteriophage T3 (Agritope, 1996)
    b. Fragment of aminocyclopropane carboxylic acid synthase gene from tomato (DNA Plant Technology, 1994)
    c. Aminocyclopropane carboxylic acid deaminase gene from *Pseudomonas chloraphis* strain 6G5 (Monsanto, 1994)
2. Delayed Softening
    a. Fragment of polygalacturonase gene from tomato (Zeneca Plant Sciences, 1994

C. New Oilseed Rape Varieties

1. Male Sterile/Fertility Restorer
    a. Barnase (male sterile) and barstar (fertility restorer) from *Bacillus amyloliquefaciens* (Plant Genetics System, 1996)
2. Glufosinate-Tolerant (two lines)
    a. Phosphinothricin acetyltransferase from *Streptomyces viridochromogenes* (two consultations by AgrEvo, 1995 and 1997)
3. Laurate Canola
    a. 12:0 acyl carrier protein thioesterase from *Umbellularia californica* (Calgene, 1995)
4. Glyphosate-Tolerant
    a. Enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (Monsanto, 1995)

D. New Cotton Varieties

1. Sulfonyl Urea-Tolerant
    a. Acetolactate synthase from *Nicotiana tabacum* (Du Pont, 1996)
2. Glyphosate-Tolerant
    a. Enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (Monsanto, 1995)
3. Insect-Protected
    a. CryIA(c) from *Bacillus thuringiensis* subsp. *kurstaki* (Monsanto, 1995)
4. Bromoxynil-Tolerant
    a. Nitrilase gene from *Klebsiella ozaenae* (Calgene, 1994)

E. New Potato Varieties

1. Insect-Protected
    a. CryIIIA from *Bacillus thuringiensis* (two consultations by Monsanto, 1994 and 1996)

F. New Soybean Varieties

1. High-Oleic Acid
    a. Sense suppression of endogenous Delta-12 desaturase (GmFad2-1) gene (Du Pont, 1997)
2. Glyphosate-tolerant
    a. Enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (Monsanto, 1994)

G. New Squash Varieties

1. Virus-Resistant
    a. Coat protein genes of cucumber mosaic virus, zucchini yellow mosaic virus, and watermelon mosaic virus 2 (Seminis Vegetable Seeds, 1997)
    b. Coat protein genes from watermelon mosaic virus 2 and zucchini yellow mosaic virus (Asgrow Seed, 1994)

H. New Papaya Variety

1. Virus-Resistant
    a. Coat protein gene from the ringspot virus (University of Hawaii and Cornell University, 1997)

I. New Radicchio Variety

1. Male Sterile
    a. Barnase from *Bacillus amyloliquefaciens* (Bejo Zaden, 1997)

III. Antibiotic Resistance Markers Seen in Consultations to Date

The FDA understands that crop developers are seeking alternative markers for selection of transformants that have the desired trait. Methods are also being developed to excise out antibiotic marker genes after they have been used in the selection process (Dale and Ow, 1991; Ebinuma, 1997). However, development of many of the transgenic plants that are on the market and those that are approaching commercialization was initiated

several years ago at which time antibiotic resistance selection methods were widely used. Thus, at the preser time, and for the foreseeable future, there will be a need to address the safety of the use of antibiotic resistance marker genes in transgenic crops used for food or animal feed.

Although many types of selectable markers, including antibiotic resistance genes, herbicide tolerance genes, metal tolerance genes, genes involved in amino acid metabolism, genes influencing phytohormone production, and screenable or reporter genes, are used in the development of transgenic crops (WHO, 1993; Karenlampi, 1996), by far the most prevalent are those conferring resistance to antibiotics. Antibiotic resistance was used as a selectable marker in 31 out of 52 consultations the agency has had regarding transgenic crops to date. Neomycin phosphotransferase II (nptII, APH(3')-II) accounted for 27 of these cases, and hygromycin phosphotransferase (aphIV, hpt) accounted for the rest.

In addition to markers that are used for selection, transgenic crops frequently have antibiotic resistance markers under bacterial promoters incorporated into their genome. These markers are necessary for passage of the constructs in bacterial systems, and most often end up in the genomes of transgenic plants because biolistics or gene gun methods using the entire construct are used to transform the plant cells. However, because the genes are under the control of bacterial promoters, they are functional only in the prokaryotes that were used in developing the gene constructs; they are not expressed in the plant even though the entire gene or multiple copies of the gene may be present.

In the 52 consultations the agency has had to date regarding plants developed using rDNA technology, there have been 19 consultations where the transgenic crop has one or more antibiotic resistance marker genes under a bacterial promoter incorporated into the plant genome (*beta*-lactamase (bla) in 12, aminoglycoside adenyltransferase (aad) in 4, nptII in 3, neomycin phosphotransferase III (nptIII) in 1, chloramphenicol acetyltransferase gene (cat) in 1, and a tetracycline resistance gene (tet^R) in 1). In some cases, only partial fragments of the genes were incorporated.

By the end of 1997, 30 out of 52 consultations that were initiated by developers have been completed. Table lists the antibiotic resistance markers found in the transgenic plants (both expressed and those under bacterial promoters). The consultations are arranged by crop.

Antibiotic Resistance Markers in Completed Consultations through 1997

Crop	No. of Consultations	Antibiotic Resistance Markers Used for Selection	Antibiotic Resistance Genes under Bacterial Promoters
Corn	9	---	3 *nptII* 5 *bla*(9) 1 *cat*(10)
Tomato	4	4 *nptII*	---
Oilseed Rape	5	3 *nptII*	---
Cotton	4	3 *nptII*	2 *aad*
Potato	2	2 *nptII*	1 *aad*
Soybean	2	1 *nptII*(11)	1 *bla*
Squash	2	2 *nptII*(12)	---
Papaya	1	1 *nptII*	*tetR*(13)
Radicchio	1	1 *nptII*	---

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V. Notes

(9) The -lactamase gene in one of the lines is truncated.

(10) The chloramphenicol acetyl transferase gene is truncated and rearranged.

(11) Although nptII was used for selection, the gene segregated out in subsequent generations and the

modified soybean variety on which consultations were concluded did not contain nptII.

(12) In one of the two transgenic squash lines, nptII was used for selection but gene subsequently segregated out. Thus the new squash variety that was the subject of a consultation did not contain nptII.

(13) Only a partial fragment of the tetracycline resistance gene is present.

Appendix 3. Review of Positions by Other Government Agencies and International Bodies on Antibiotic Resistance Marker Use in Transgenic Plants

I. The U.S. Environmental Protection Agency

Pesticides are regulated by the Environmental Protection Agency (EPA) under the Federal Insecticide, Fungicide, and Rodenticide Act and under section 408 of the Federal Food, Drug, and Cosmetic Act. Thus, in the case of a new variety that has been modified to have a pesticidal trait, the safety of the pesticide, as well as the genetic material needed to express the pesticide, and marker genes used to confirm the presence of the pesticidal substance are evaluated by the EPA. EPA sets tolerances for pesticides and pesticide inert ingredients or exempts them from tolerance requirements. In the *Federal Register* of September 28, 1994, EPA established an exemption from the requirement of a tolerance for residues of neomycin phophotransferase II and the genetic material necessary to produce it when produced in plants as a plant pesticide inert ingredient (U.S. EPA, 1994).

II. The U.K. Advisory Committee on Novel Foods and Processes

The Advisory Committee on Novel Foods and Processes (ACNFP) of the United Kingdom's Ministry of Agriculture Fisheries, and Foods recently published a document entitled "The Use of Antibiotic Resistance Markers in Genetically Modified Plants for Human Food: Clarification of Principles for Decision-Making" (ACFNP, 1996). In that document, the ACNFP stated that the evaluation of food/feed from genetically modified plants containing antibiotic resistance marker genes will be conducted on a case-by-case basis and will be determined to a large extent by considerations of the possibility of the transfer, maintenance and expression of the marker gene in gut or rumen microorganisms, the clinical use and importance of all the antibiotics for which resistance is encoded and the potential toxicity or allergenicity of the gene product. Since the ACNFP considers the safety issues associated with the antibiotic resistance markers on a case-by-case basis, it does not publish prescriptive lists of "acceptable" and "unacceptable" antibiotic resistance marker genes.

The ACNFP considers that there is a finite, albeit a very low probability of transfer, maintenance and expression of the gene in rumen or gut microorganisms. ACNFP considers this of little concern for antibiotic resistance markers with plant regulatory sequences but not those with bacterial regulatory sequences. It states that where transfer is considered to be possible and subsequent expression likely and the antibiotic which would be affected is of major clinical importance, *e.g.*, ampicillin, use of the marker would be unlikely to be approved. With respect to toxicity and/or allergenicity of the gene product, the ACNFP believes that thorough characterization of the antibiotic resistance marker gene allows the comparison of sequence data with genes known to code for toxic or allergenic gene products. In addition, *in vitro* degradation studies in gastric fluid can be used to indicate the likelihood for persistence of the gene product in the digestive tract and provide further reassurance about the safety of the antibiotic resistance marker gene.

Recently, the ACNFP recommended that approval be denied for use of unprocessed insect-resistant corn developed by Ciba-Geigy for animal feeds (Letter from the Ministry of Agriculture, Fisheries and Foods, to Ciba-Geigy Limited, April 29,1996). This transgenic corn contains an intact *beta*-lactamase gene with a promoter and an origin of replication (*ori*) derived from the pUC18 vector. Unlike the *ColE1 ori* vector found in nature which generates 4 to 18 copies per cell, the pUC *ori* generates over 600 copies per cell. The ACNFP believed that there is a finite risk of transfer of the *beta*-lactamase gene from the transgenic corn to bacteria in the rumen or gut of livestock and that as a consequence of the presence of a promoter and the pUC ori on the *beta*-lactamase gene, such transfer would have extremely grave consequences for therapy with *beta*-lactam antibiotics(14).

III. The European Commission Scientific Committees for Food, and Animal Nutrition

In December, 1996, the European Union's Scientific Committee for Food (EC/SCF) issued an opinion regarding the potential for adverse health effects from the consumption of genetically modified maize (modified for insect resistance through insertion of the Bt-endotoxin gene, and for increased tolerance to the herbicide glufosinate ammonium) including any potential adverse effects from the non-expressed *beta*-lactamase gene (EC/SCF, 1996). The committee concluded that the possibility that the product would add significantly to the already widespread occurrence of ampicillin resistant bacteria in animals and man is remote. This conclusion was based on evidence derived from theoretical considerations, laboratory studies, a step-wise assessment regarding the gene construct itself, its distribution and persistence in maize and products derived from this crop, the possibility of transfer of the gene from maize to gram negative bacteria, and the possibility that it would function in such bacteria. All of this evidence led to the conclusion that the risk of bacterial transformation is extremely low. Further, the committee concluded that even if transfer were to take place, it would have no

detectable additional effect as the *beta*-lactamase gene is already widespread in nature including human and animal gastrointestinal tracts. The committee stated that it proposes to scrutinize the future needs and applications of antibiotic resistance marker genes.

Similarly, with respect to the presence of the *beta*-lactamase gene, the European Commission's Scientific Committee for Animal Nutrition (EC/SCAN) concluded that the probability of the transfer of a functional gene construct from the genetically modified maize into bacteria is virtually zero, and that if the virtually impossible event occurred, it would have no clinical significance. There is no evidence of a risk of causing *beta*-lactam antibiotic resistance in the animal gut bacteria from the use of the genetically modified maize (EC/SCAN, 1996)

IV. The Nordic Working Group on Food Toxicology and Risk Assessment

The Nordic Working Group on Food Toxicology and Risk Assessment under the auspices of the Nordic Council o Ministers issued a document entitled " Health Effects of Marker Genes in Genetically Engineered Food Plants" i 1996 (Karenlampi, 1996). The report deals with the food safety aspect of genes conferring resistance to the antibiotics kanamycin, hygromycin and streptomycin, and to the herbicides glufosinate, glyphosate and chlorsulfuron as well as the *gus* reporter gene. Based on the detailed safety analysis that has been conducted to date, it suggests a positive list of marker genes acceptable in the genetic engineering of food plants, namely the *kan*^{r} gene and the glyphosate tolerance marker gene encoding 5-enolpyruvylshikimate-3-phosphate synthase. It also suggests that the glufosinate tolerance gene encoding phosphinothricin acetyltransferase might be the next marker gene to be added to such a list.

V. World Health Organization/Food and Agriculture Organization

The World Health Organization (WHO) has conducted a workshop on health aspects of marker genes in genetically modified plants (WHO, 1993). The workshop recognized the need for marker genes and that it was impractical at present to remove marker genes from modified plants once they have fulfilled their function. The WHO workshop concluded that many of the general safety issues raised about other genes apply to marker genes as well. These include potential toxicity and allergenicity of the gene product and possible secondary and pleiotropic effects of the insertion. The workshop concluded that in assessing the safety of the proteins expressed by marker genes, the focus of the assessment should be on the function of the protein rather than its structure. It also concluded that there is no reason to suppose that marker gene proteins pose a particular allergenic concern but that, if the gene is obtained from a source known to cause allergy, the allergenicity of the gene product should be investigated. The workshop concluded that in addition to safety issues common to all introduced proteins, there are safety issues specific to antibiotic resistance markers. These include potential inactivation of an oral dose of antibiotic due to the presence of a marker gene product in food, and horizontal transfer of the gene to gut microorganisms.

The workshop concluded that there is no recorded evidence for transfer of genes from plants to microorganisms in the gut and that if transfer did occur, any health concern would depend on many factors, including the ability of the transformed microorganisms to replicate in the gut and to express the gene product

This conclusion was reaffirmed in the 1996 joint Food and Agriculture Organization (FAO)/WHO Expert Consultation on Biotechnology and Food Safety (WHO, 1996), which stated that the most relevant food safety issue concerning gene transfer is the potential consequence of the transfer of an introduced gene from material derived from a genetically modified organism to microorganisms in the gastrointestinal tract, in such a way that the gene can be successfully incorporated and expressed, and impact human or animal safety. The consultation added that there is no recorded evidence for the transfer of genes from plants to microorganisms in the gut and that there are no authenticated reports of such bacterial transformation in the environment of the human gastrointestinal tract.

The consultation cited the events that would need to occur for gene transfer - survival of the DNA in the hostile environment of the GI tract, need for recipient organisms to be transformation-competent, need for the DNA to bind to the recipient organism and translocate across the cell membrane, survival of the DNA from degradation by the restriction/modification system of the microorganism DNA, and need for the DNA to integrate into the host genome or plasmid which requires sequences homologous to the host DNA at both ends of the foreign DNA- and concluded that the possibility of gene transfer is vanishingly small. The consultation further concluded that data on such transfer will only be needed when the nature of the marker gene is such that, if transfer were to occur, it would give rise to a health concern. In assessing any potential health concerns, the human or animal use of the antibiotic and the presence and prevalence of resistance to the same antibiotic in gastrointestinal microflora should be considered. As an example, the consultation noted that the antibiotic vancomycin is critical in the treatment of certain bacterial diseases where multiple antibiotic resistance is prevalent, and there is lack of alternatives.

VI. References

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7. U.S. Environmental Protection Agency, "Neomycin Phosphotransferase II; Tolerance Exemption," *Federal Register*, 56, 49351, 1994.
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VII. Note

(14) On the other hand, in a conference sponsored by Tufts University and the Foundation for Nutritional Advancement (1996), a group of scientists discussed Ciba-Geigy's transgenic corn containing the *beta*-lactamase gene and concluded that use of this particular type of *beta*-lactamase gene constitutes an insignificant to near zero risk of causing ampicillin resistance in either animals or humans because 1) the probability of DNA survival in segments large enough to be taken up by bacteria is very low, 2) the probability of bacteria taking up or incorporating DNA into the bacterial genome is virtually zero, and 3) even if the DNA from the marker gene was to be incorporated in the bacterial genome, there is a low probability that it would be expressed. In addition, they concluded that the clinical significance is virtually zero because it is ubiquitous, already occurs in nature, and can be overcome by antibiotics other than ampicillin.

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