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Through: Director,
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TO: CFSAN Biotechnology Coordinator, HFF-300
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SUBJECT: Use of Kanamycin Resistance Markers in Tomatoes.

On October 5, 1992 a consult was received by the Division of Anti-Infective Drug Products requesting that we provide an opinion on the effect that utilization of the kanamycin resistance marker, aminoglycoside phosphotransferase (3') Type II (APH (3') II), may have on clinically important antibiotics when used in transgenic tomato plants. Specifically, we were asked to address what is considered to be the principle but unresolved safety issue. The issue is "Whether the APH(3')II protein can impair the clinical efficacy of orally administered susceptible antibiotics".

The sponsor performed a number of in vitro studies with the aminoglycoside phosphotransferase (APH) which were designed to answer questions on 1) the fate of the protein in the gastrointestinal tract of persons consuming tomatoes and 2) on the ability of the APH (3') II to phosphorylate aminoglycosides under conditions found in the gastrointestinal tract. Thorough reviews of this data have been completed by others and since I concur with most of their conclusions, I will not review that data further. This includes: 1) the amino acid sequence homology studies designed to assess homology with known toxins or allergens, 2) kan used in gene therapy, 3) the toxicity study, and 4) the studies on the fate of APH and implications for kanamycin efficacy. The bioassays used in the later studies were not addressed because: 1) the measurement of zone diameters 8 to 12 hours post incubation is not consistent with standard practice (18 hrs incubation time), 2) inocula densities were not provided, and 3) media used is not normally used for disc diffusion assays. In summary, I agree
with previous reviews suggesting that the studies appear to provide evidence that APH (3') II gastrointestinal tract concentrations will not pose a safety problem during therapy by inactivating aminoglycoside antibiotics.

In addition, the sponsor contends that resistance to kanamycin already exists in microorganisms colonizing the gastrointestinal tract and utilization of the kan marker in transgenic tomatos will not increase the genetic burden in that environment. Thus safety will not be compromised by the use of this marker in their transgenic tomato plants. A review of their citation (Atkinson, B.A., Species Incidence and Trends of Susceptibility to Antibiotics in the United States and Other Countries: MIC and MBC) reveals that the basis for this conclusion is based on MIC/MBC studies that reveal little, if anything, regarding the mechanism of resistance. Resistance as measured by this method does not distinguish between cell permeability, target site modification, enzymatic in activation of the antibiotic, etc. So the sponsors statement that the dissemination of a gene that confers resistance to aminoglycosides by a mechanism of aminoglycoside phosphorylation is not valid.

The question that looms in the mind of this reviewer is more of a global nature: "What is the consequence of disseminating billions of copies of aminoglycoside phosphotransferase open reading frames over the surface of this country?" This is exactly what we are proposing to do. We can not predict what the consequences of this action will be. We are attempting to do so by assuming that we can ask all of the correct questions and answer all of these questions thus allowing us to make the correct assessment. With the exception of some gram negative organisms, we know very little about the evolution and the requirements for dissemination of antibiotic resistance determinants.

It has been argued that expression of this gene can not occur because it lacks the essential regulatory elements (transcriptional and translational ?) required for expression in procaryotic organisms. The T-DNA, that fragment of DNA that is transposed from the donor plasmid found in Agrobacterium tumefaciens to the recipient plant, is approximately 7.5 kilobase pairs (kbp) in length and has been sequenced according to the information that we have received. Apparently no one seems to know whether transcriptional/translational sequences reside within this fragment. Does this 7.5 kbp fragment contain procaryotic transcriptional and/or translational sequences? This is important because each transgenic plant has 2 to 10 of these 7.5 kbp fragments that, upon denaturing during replication or transcription, form stem-loop and clover-leaf structures that can undergo deletion formation or rearrangements. If transcriptional/translational sequences exist within the 7.5 kbp fragment, such rearrangements could conceivably end up proximally to the kan ORF resulting in APH (3') II expression in procaryotic systems. Further it has been demonstrated in transposon Tn21 that an integron exists which is composed of a "variable region" that allows the integration of antibiotic "cassettes" that do not contain their own promoters and are transcribed from Pout in the 5' common segment of the integron. The only requirement placed on the "cassette" is that it contain a "59-base pair" (bp) element (which have now been shown to vary in length and
sequence) and a 7-bp core site which functions in recombination resulting in insertion/deletion of the cassette. How ORF's acquire the "59-bp" element or the core has not been determined. These are but two examples of how nature can copy with the expression of DNA that does not have its own transcriptional/translational elements.

Also, it has been stated that transformation of the resident flora of the gastrointestinal tract can not occur, at least at any significant frequency, to make much difference in the overall pattern of resistance known to exist in this environment. This statement assumes that we have adequately characterized the mechanisms by which procaryotic organisms acquire DNA in nature, incorporated it and regulate its expression. In my opinion, nothing could be further from the truth. The fact that we can not demonstrate transformation in nature may be a result of our technological limitations and not because it does not occur. The fact that a gene does not contain a regulatory region does not imply that it may not be expressed in procaryotes.

In my opinion, the benefit to be gained by the use of the kanamycin resistance marker in transgenic plants is outweighed by the risk imposed in using this marker and aiding its dissemination nation wide. If we allow this proposal, we will be adding a tremendous quantitative load of genetic material to the environment which will probably assure dissemination of kanamycin resistance.

Other markers (color and light elicitors) are available and should be used but are not as easily monitored. Ease of use is the primary reason that antibiotic selection is preferred. A practice that should be discouraged when these markers are to be disseminated into the environment.

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