ENVIRONMENTAL ASSESSMENT

AND

FINDING OF NO SIGNIFICANT IMPACT

This document gives notice that the Department intends to issue a permit for release into the environment of a regulated article under regulations issued pursuant to the Federal Plant Pest Act and the Plant Quarantine Act. The permit is for a controlled field test of genetically engineered tomato plants to be conducted by the DNA Plant Technology Corporation, on a small test plot on agricultural land in Contra Costa County, California. The request for a permit has been thoroughly reviewed with a finding that there is no significant risk of introduction or dissemination of a plant pest from conducting this test as described by the DNA Plant Technology Corporation. This document also contains an Environmental Assessment and Finding of No Significant Impact on the environment relative to the field testing of the genetically engineered tomato plants.

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Permit Number 91-079-01: tomato; antifreeze gene; staphylococcal Protein A

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ENVIRONMENTAL ASSESSMENT

I. PURPOSE AND NEED

1.1 Summary

This Environmental Assessment (EA) presents scientific data and other information evaluated by the Animal and Plant Health Inspection Service (APHIS), U.S. Department of Agriculture (USDA), prior to issuing a permit for the introduction of an article regulated under Title 7 Code of Federal Regulations Part 340.

A permit, number 91-079-01, was requested by the DNA Plant Technology Corporation, Oakland, California, for a controlled field test of genetically engineered tomato to be carried out on a small test plot on agricultural land in Contra Costa County, California. The tomato plants have been modified by incorporating a synthetic antifreeze gene modeled from one isolated from the winter flounder, Pseudopluronectus americanus. This gene encodes a fusion protein which when expressed may lower the threshold temperature at which freezing damage to the plant occurs. This EA is intended to provide documentation of the APHIS review and analysis of data in which a determination was made that this limited field trial does not pose a risk of introduction or dissemination of a plant pest and will not have a significant impact on the quality of the human environment.

1.2 Finding of No Significant Impact

APHIS has determined that this limited field trial, authorized by the issuance of permit number 91-079-01, will not pose a risk of the introduction or dissemination of a plant pest and does not present a significant impact on the quality of the human environment. This Finding of No Significant Impact (FONSI) is based on the following factors:

1. A synthetic gene that encodes an antifreeze protein fused to Staphylococcus aureus Protein A (Protein A-Antifreeze Fusion protein or AFF) has been inserted into the tomato chromosome. In nature, chromosomal genetic material from plants can only be transferred to other sexually compatible plants by cross-pollination. In this field trial, the introduced genes cannot spread to another plant because the test plot is located at a sufficient distance from any sexually compatible plants with which these experimental tomato plants could cross-pollinate. Therefore, the introduced gene will be prevented from spreading to other plants by cross-pollination.

2. Neither the introduced AFF gene itself, nor its gene product, confers on tomato any plant pest characteristics. Traits that lead to weediness in plants are polygenic traits and cannot be conferred by adding a single gene.
3. The organisms, P. americanus and S. aureus, from which parts of the chimeric gene were derived are not plant pathogens.

4. The vector used to transfer the AFF gene to tomato plants has been evaluated for its use in this specific experiment and does not pose a plant pest risk. The vector, although derived from a DNA sequence with known plant pest potential, has been disarmed; that is, genes that are necessary for producing plant disease have been removed from the vector. The vector has been tested and shown not to be pathogenic to any susceptible plants.

5. The vector agent, the bacterium that was used to deliver the vector DNA and the AFF gene into the plant cells, has been shown to be eliminated and no longer associated with the transformed tomato plants.

6. Horizontal movement of the introduced gene is not known to be possible. The vector acts by delivering and inserting the gene into the tomato genome (i.e., chromosomal DNA). The vector does not survive in the transformed plants. No mechanism that can transfer an inserted gene from a chromosome of a transformed plant to a chromosome of another organism has been shown to exist in nature.

7. The gene product, AFF protein, acts by inhibiting ice crystalization. The only possible phenotypic change in these transgenic plants is a decreased susceptibility to freeze damage. Effects on complex agronomic traits such as yield are not expected.

8. DNA sequences used to regulate expression of the inserted genes in tomato are derived from the plant pest Agrobacterium tumefaciens and the cauliflower mosaic virus. These sequences in themselves, however, encode no proteins, and confer no plant pest related property on the recipient plants.

9. The test is to take place on a small field site, approximately 0.25 acre in size. The site has good security: public access is restricted, a visual barrier of corn plants will border the experimental blocks, and employees are on duty 7 days a week .

10. At the conclusion of the test, all plant material left in the field will be uprooted, allowed to desiccate, and then incorporated into the soil. The site will be monitored for any volunteer plants that may arise.

This EA and FONSI have been prepared in accordance with (1) the National Environmental Policy Act of 1969 (NEPA) (42 United States Code (U.S.C.) 4331 et seq.); (2) Regulations of the Council on Environmental Quality for Implementing the Procedural Provisions of NEPA (Title 40 CFR Parts 1500-1509); (3) USDA Regulations for implementing NEPA (7 CFR Part 1b); and (4) APHIS Guidelines for implementing NEPA (44 FR 50381-50384 (August 28, 1979) and 44 FR 51272-51274 (August 31, 1979)).

1.3 U.S. Department of Agriculture Regulations

The request for a permit was submitted pursuant to regulations published in the Federal Register on June 16, 1987 (52 FR 22892- 22915), that became effective on July 16, 1987. The regulations, "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There is Reason to Believe Are Plant Pests," have been codified in Title 7 of the Code of Federal Regulations in new Part 340. The regulations, which were promulgated pursuant to authority granted by the Federal Plant Pest Act, as amended (7 U.S.C. 150aa-150jj), and the Plant Quarantine Act, as amended (7 U.S.C. 151-164a, 166-167), regulate the introduction (importation, interstate movement, or release into the environment) of certain genetically engineered organisms and products. Under Section 340.0 of the regulations, a person is required to obtain a permit prior to introducing a regulated article. A genetically engineered organism is deemed a regulated article if the donor organism, recipient organism, vector or vector agent used in engineering the organism belongs to one of the taxa listed in the regulation and is also a plant pest. The genetically engineered tomato plants in the DNA Plant Technology submission are deemed "regulated articles." The genetically engineered tomato plants are regulated articles because the vector used to transfer the genes came from A. tumefaciens, which is widely recognized as a plant pest, and because expression of the genes introduced into tomato is directed by regulatory sequences derived from the cauliflower mosaic virus, which is a known plant pest.

1.4 Need for Field Testing of Experimental Products

Limited field releases are needed so that information can be gathered for scientific evaluation of the efficacy of the genetic change. The plants have been tested in the greenhouse to obtain initial data relating to the genetic stability of the plants and preliminary data on efficacy. It is normal for controlled field tests to be performed after greenhouse testing to confirm the efficacy data, which can only be validated in the environment using standard agricultural practices. Such limited field testing is required to develop a potential agricultural product.

II. BACKGROUND

This EA presents scientific data and other information evaluated by APHIS, USDA, prior to issuing a permit for the introduction of an article regulated under the 7 CFR 340 (52 FR 22892-22915, June 16, 1987). This EA describes the information that was evaluated in determining whether to issue DNA Plant Technology a permit for a planned field test trial of genetically engineered tomato.

The recipient organism is tomato, Lycopersicon esculentum, which has been modified by the insertion of two genes. One is a chimeric gene, that encodes a Protein A-Antifreeze fusion protein that may lower the temperature at which freezing damage of the plant tissue is initiated. Two organisms are the source of the fusion protein gene: the winter flounder, P. americanus, and the common bacterium, S. aureus. The second gene is a marker gene conferring resistance to the antibiotic kanamycin, referred to as the neomycin phosphotransferase gene (NPT II). This gene was incorporated into tomato only to facilitate identification of the transformed plants, and confers no agronomically significant property on those plants.

In the sections that follow, we first describe the alternatives available to APHIS. In subsequent sections, we delineate the field plot design, field test protocol, and other factors necessary to identify the aspects of the environment that would potentially be affected. We examine in detail the biology of each component of the genetically engineered organism: the recipient plant, the donor organism, and the bacterium that was used to transfer the genes into the plant. We attempt to identify the potential impacts to the environment inherent in each of those components and describe the ways in which the risk to the environment is limited either by the nature of the organism or by specific safeguards that have been designed into the protocol. In the final section, we state our conclusion that no significant impact to the quality of the human environment will result from issuing the permit described in this EA.

II. ALTERNATIVES

The regulations in 7 CFR 340 set forth the conditions under which a permit is required and identify the responsibilities of APHIS in responding to a request for a permit. Under Section 340.3(b), APHIS has 120 days to process a permit for introduction that is deemed by the Agency to be complete. APHIS is faced with two alternative actions after a permit application is deemed to be complete. Section 340.3(e) provides:

> "... A permit shall be granted or denied. If a permit is denied, the applicant shall be promptly informed of the reasons why the permit was denied and given the opportunity to appeal the denial in accordance with the provisions of paragraph (g) of this section. If a permit is granted, the permit will specify the applicable conditions for introduction of the regulated article under this part."

These two alternatives are discussed in the following two sections.

3.1 Alternative 1: Issue a Permit for the Introduction (Release into the Environment) of a Regulated Article

One alternative is to issue a permit for the introduction of a regulated article. The permit would allow the applicant to conduct a limited field test as proposed in the permit request. To issue a permit for the introduction of a regulated article pursuant to 7 CFR 340, APHIS must find that there is no significant risk of introduction or dissemination of a plant pest due to the permitted activity under the specified conditions (7 CFR 340.3(e)). APHIS may specify conditions in addition to those included in the permit submission, if such conditions are necessary to prevent dissemination of a potential plant pest.

3.2 Alternative 2: Deny a Permit

APHIS must deny the permit if the proposed field test would present a risk of introduction or dissemination of a plant pest that is new or not widely prevalent. If a permit is denied, the applicant must be fully informed by APHIS of the reasons for the denial. The applicant has a right to appeal the denial of a permit (7 CFR 340.3(g)).

IV. DESCRIPTION OF RECIPIENT, DONOR, AND VECTOR

As stated in the preamble to APHIS' regulations in 7 CFR 340 (52 FR 22892-22915), the regulations do not regulate an article merely because of the process by which it was produced, but regulate certain genetically engineered organisms and products that present some potential for plant pest risk. APHIS has determined that it is important to evaluate genetically engineered organisms that have been engineered using a recognized plant pest as the recipient organism, or as the source of inserted genes (donor), and also, those that use vectors or vector agents that are, or are from, pest organisms. In this case, the tomato plants were genetically engineered using a vector system derived from the plant pathogenic bacterium, A. tumefaciens.

In the sections that follow, we have examined the biology of the recipient plant, the donor organism, and the bacterium used to transfer the genes into the plant. We have focused on the potential impacts to the environment inherent in each of those components. Specifically, we describe the ways in which the risk to the environment is limited either by the nature of each of the organisms or by specific safeguards that have been designed into the field test protocol.

4.1 Recipient Related Impacts

In this section of the EA, the potential impacts to the environment from the introduction of genetically engineered tomato are discussed. The biology of tomato and plants related to tomato is considered. Because the mechanism by which genes are commonly moved from one plant to another in nature is through cross-pollination with sexually compatible plants, the plants with which tomato can cross-pollinate are described. The methods by which tomato is commonly cultivated are examined to identify whether any pest characteristics such as weediness are newly expressed in the transformed plants. Other potential impacts from the tomato plants are also analyzed in this section.

4.1.1 Origin of the Cultivated Tomato

The commercial tomato belongs to a species referred to as L. esculentum. Lycopersicon is a relatively small genus within the large and diverse family Solanaceae, which contains approximately 90 genera. The genus is currently thought to consist of cultivated tomato, L. esculentum, and eight closely related wild Lycopersicon species (Rick, 1976). Lycopersicon species are native to Ecuador, Peru, and the Galapagos Islands; however, most evidence suggests that the site of domestication was Mexico (Taylor, 1986).

4.1.2 Propagation and Cultivation of Tomato

Tomato is a highly specialized crop bred to be grown under intensive monoculture. Tomato is grown commercially wherever agronomic conditions will permit an economic yield to be obtained. California and Florida are the leading producers of fresh market tomatoes in the United States; California is the leader in producing processing tomatoes. Commercial tomato cultivars are self-compatible and self-pollinated when field grown, but the flowers can be manipulated for efficient yields of hybrid seed. As is true for most self-pollinating plants, the viability of exposed tomato pollen is limited. The distance required between certified seed fields, in practical terms the effective distance tomato pollen can travel under field conditions and remain viable (Anonymous, 1971; Rick, 1976), is 30 feet.

4.1.3 Tomato Does Not Cross-pollinate Other Plant Species

The factors that prevent cross-pollination are well documented and are applicable to genetically engineered tomato. Tomato can be crossed by hand-pollination to all wild Lycopersicon species with varying degrees of success. The genus has been divided into two subgenera, the esculentum complex which contains those species which are easily crossed with commercial tomato and the peruvianum complex which contains those species which are crossed with

considerable difficulty (Stevens and Rick, 1986; Taylor, 1986). Hybridization between members of the two subgenera usually leads to early embryo breakdown and inviable seed. Hybrids are generally only produced using specialized laboratory techniques.

The closest genetic relatives of tomato are in the genus Solanum. Hybrids have been obtained between L. esculentum and S. lycopersicoides, but these hybrids are usually sterile (Stevens and Rick, 1986). No other member of the genus, including S. nigrum, a common weed in tomato fields, has yielded any viable hybrids with tomato (Taylor, 1986).

There is no evidence that tomato plants can cross-pollinate with other plants in the area of the field test. Similarly, there is no evidence that the engineered tomato plants will behave differently from the parental line with respect to cross-pollination. Thus, there is no risk of the spread or establishment of the AFF gene from any of the engineered tomato plants to any other related or unrelated plant species.

4.1.4 Dissemination of Transformed Tomato Plants

The transformed tomato has gained no measurable selective advantage over the nontransformed parental strain in its ability to be disseminated or to become established in the environment.
The risk of horizontal movement of the newly introduced genes from the genetically engineered tomato plants into the environment is negligible because the genes are incorporated into the plant genome. No mechanism is known to exist in nature capable of transferring the genetic material from the tomato genome to another organism with which tomato does not naturally hybridize.

4.2 Donor Related Impacts

In this section of the EA, the potential impacts to the environment from the introduction of the AFF gene are discussed. The procedure by which the gene was isolated is summarized. Another gene, used only as a marker in the transformation procedure, is also described. Whether any unique risks to the environment might be posed by introducing tomato containing the AFF gene into the environment is considered.

4.2.1 The antifreeze gene

The tomato plants constructed by The DNA Plant Technology Corporation contain a gene based on one identified in the winter flounder, P. americanus, that encodes the an antifreeze protein. The purpose of inserting this gene into tomato plants is to limit the damage that normally occurs to tomato fruit when frozen.

Antifreeze proteins have been isolated from the blood of a number of marine fishes that inhabit temperate oceans and near-shore waters of north temperate oceans (DeVries, 1983). They act by lowering the freezing point and thus allow the survival of these fish in frigid temperatures. The purpose of this experiment is to determine if the expression of an antifreeze protein will prevent tissue damage and the changes in flavor, texture, and color in tomato fruit that result. Ice formation damages plant tissue by damaging the membrane network of the cell leading to release of degradative enzymes bound within these membranes and the loss of integrity of the plasma membrane. The damage done by the initial ice crystal formation is enhanced by crystal growth due to recrystalization. Antifreeze proteins inhibit the initial crystal formation and recrystilization. Damage is also induced by crystal growth extracellularly primarily by dehydration. DNA Plant Technology has inserted two antifreeze constructs into these two tomato plants, one directing the protein to remain intracellular and a second that encodes a secretory protein localized external to the cells in which it is synthesized.

There are four major classes of antifreeze proteins that can be distinguished by their helicity, amino acid composition, size, and the presence of sugar residues (Davies and Hew, 1990). The winter flounder contains two AFP's which are prototypes of Type I. Type I peptides typically contain three repeating units of the following motif: $ThrX_2AsxX_7$ where thr is threonine, X is alanine

or another amino acids that favors alpha-helix formation and asx is aspartate (Davies and Hew, 1990; Devries and Lin, 1977). Antifreeze peptides within the type I class range in molecular weight from 3000 to 5000 daltons (Davies and Hew, 1991).

A gene from winter flounder that encodes an antifreeze protein has been isolated and sequenced. It is synthesized as an 82 amino acid precursor protein from which the 38 amino acid long mature protein is cleaved. The precursor protein contains a signal polypeptide and the 38 amino acid prosequence (Davies et al., 1982). The function of signal sequences are to allow secretory peptides to be exported from their site of synthesis to their site of action (see section 4.3.4). In the case of the antifreeze proteins, synthesis takes place in the liver and they are secreted into the circulatory system (DeVries, 1983; Davies et al., 1982).

From the nucleotide sequence of the antifreeze gene, an amino acid sequence has been deduced which agrees well with that reported by direct sequencing of the protein (Davies et al., 1982; Davies and Hew, 1991; DeVries and Lin, 1977). The 38 amino acid peptide contains three repeats of the following motif: Thr-Ala-Ser-Asx-Ala_6-Leu where ala is alanine, ser is serine, and leu is leucine (Davies et al., 1982).

Antifreeze proteins exert their effect by depressing the freezing point of water. They have been shown to adsorb onto the surface of ice crystals and cause a realignment of the axis around which the crystal is formed (Davies and Hew, 1991). The adsorption of the peptide onto the surface plane of the ice crystal is believed to be responsible for the lowering of the freezing point and prevention of recrystilization. The exact mechanism by which these effects occur is unknown but are likely due to the structures, amino acid sequences, and molecular weights of the peptides. The alpha-helicity of the peptide aligns the polar amino acids (threonine and aspartate) on one side of the molecule and the non-polar amino acids (alanine and leucine) on the opposite side. The spacing of threonine and aspartate resulting from this conformation matches the spacing of the oxygen atoms of water in an ice crystal. This match allows hydrogen bonding to occur between the side chains of the amino acid residues and the oxygen atoms of water molecule (DeVries and Line, 1977; DeVries, 1983; Davies and Hew, 1991). The motif in which their is a long stretch of non-polar amino acids that separate the water binding amino acid residues is believed to be necessary to impede ice nucleation and thus recrystilization (Davies and Hew, 1991; Devries and Lin, 1977; DeVries 1983). There is a critical number of repeating units needed for functioning. Shortening the winter flounder peptide which contains 3 repeating units by one, inactivates it (Davies and Hew, 1991).

The expression of antifreeze proteins have been successfully engineered in heterologous systems. The wolfish antifreeze protein was fused to appropriate regulatory elements and expressed in the fruit fly. An active peptide was secreted and properly processed in this organism (Rancourt 1990).

DNA Plant Technology has synthesized a Type I antifreeze gene based on the winter flounder gene. It contains 5 units of the repeating motif described above. This gene was fused at its 5' end with a truncated form of the Staphylococcus aureus Protein A gene to yield a fusion protein in which the Protein A is fused to the amino-terminus of the synthetic antifreeze peptide. Protein A binds IgG molecules (Nilsson et al., 1985) thus facilitating the purification of the peptide from the tomato plants. In other words it functions as a marker to identify the polypeptide in tomato. Another advantage of the fusion protein is that since it is larger, 35 kilodaltons, than the antifreeze peptide alone, it is potentially more stable in the plants. The gene encoding the 35 kilodalton product is referred to as AFF-1. This construct is located intracellularly. A second construct encodes a secretory form of the protein. The signal sequence of the PR1b gene isolated from tobacco is fused 5' to the Protein A sequence. The fusion of the PR1b sequence with the AFF gene results in a chimeric construct which produces a precursor protein that has the PR1b signal sequence fused to the N-terminus. The mature protein that is processed from the precursor protein is localized in the extracellular space (see section 4.3.4). This gene is referred to as AFF. Two independent transformants of each genotype were chosen for field testing. The plants to be tested were selected on the extent of expression

of the fusion gene. In each case expression was determined by mRNA levels, the amount of fusion protein as measured by immunoblotting plant extracts with Protein A antibody, and antifreeze activity as measured by ice crystal growth in the presence of extracted antifreeze protein (SPLAT assay). Expression was higher in those plants expressing the secretory protein, AFF-3, and there was a strong correlation between activity, mRNA levels, and protein expression in those two plants chosen for field testing. Expression of mRNA was high in those plants containing AFF-1 but antifreeze activity was low.

The organisms from which the gene was derived, P. americanus and S. aureus, are not plant pests. The chimeric Protein A/ antifreeze genes nor their gene products have any inherent plant pest characteristics.

4.2.2 Marker Gene Used as an Experimental Control

Another gene, besides the chitinase gene, has been incorporated into chromosomal DNA during transformation. This gene encodes the enzyme neomycin phosphotransferase II (NPT II). This enzyme confers resistance to the common aminoglycoside antibiotic, kanamycin, by phosphorylating the drug and thereby inactivating it. The NPT II gene is derived from the prokaryotic transposon Tn5 (Beck et al., 1982) and functions only as a genetic marker in the initial cell selection process following transformation. Tn5 is derived from the bacterium, Klebsiella. Klebsiella is not a plant pathogen and the gene and its product do not have any inherent plant pest characteristics.

4.3 Vector and Vector Agent Related Impacts

In this section of the EA, the potential impacts to the environment from the vector system that was used to transfer the genes into plant cells are discussed. The term "vector" refers to the actual DNA molecule that carries the genes into the plant cells and facilitates their incorporation into plant chromosomal DNA. The term "vector agent" is used to denote the bacterium which transfers the vector into plant cells. In this submission, the vector is the disarmed Ti plasmid and the vector agent is A. tumefaciens. The vector is called disarmed because it can no longer incite crown galls.

The biology of A. tumefaciens and the unmodified Ti plasmid is described. The ways in which the natural A. tumefaciens system has been modified to ensure its biological safety are examined in detail. Those modifications include disarming the plasmid; i.e., making it nonpathogenic and partitioning the rest of the plasmid genes onto two separate plasmids, referred to as a binary plasmid system. This modified vector system is scrutinized to assess its environmental safety. The scientific literature is surveyed to assess the irreversibility of this system and the stability of the inserted genes in the plant genome.

4.3.1 Use of the Transformation System

The phytopathogen A. tumefaciens incites a disease called crown gall. If the bacterium is present at a wound site on a susceptible plant, a complex series of events is initiated that results in transfer of genetic information (DNA) from the bacterium to the plant and leads to gall formation (Thomashow et al., 1980; Matthysse, 1984). Many bacterial genes are required for successful infection. Some of the genes are encoded on host chromosomal DNA (chvA and chvB), while others are on a DNA plasmid called Ti (Tumor-inducing) (Douglas et al., 1985; Van Larebeke et al., 1974). Host chromosomal genes, expressed constitutively, are required for successful attachment of the bacterium to the plant at the wound site. Present in exudates of wounded plant tissues is a compound, acetosyringone, that activates a series of genes in the so-called "virulence" (vir) region on the Ti plasmid (Stachel et al., 1985). This region encodes at least seven genes (Klee et al., 1983). The products of these genes cause another DNA segment on the same plasmid, called the "transfer" region
(T-region), to be moved into plant cells (Stachel et al., 1984). Unmodified T-DNA contains as many as 13 genes depending on the particular Ti plasmid from which it was derived. Unmodified T-DNA also encodes genes responsible for the biosynthesis of opines, unusual amino acid derivatives such as nopaline and octopine, which normally provide a specialized nutrient source for

Agrobacterium growing on plant tissue. Ti plasmids generally carry genes that direct the biosynthesis of one particular class of opines, and the T-DNA regions of nopaline-type plasmids and octopine-type plasmids are not identical.

T-DNA genes only function in plants and not in the bacteria from which they were transferred. The T-region is characterized by imperfect direct repeats of a specific 25 base pair (bp) border sequence (Wang et al., 1984; Yadav et al., 1982). A sequence-specific endonuclease, encoded by one of the vir genes, nicks the T-region at the 25 bp sequence. This provides an initiation point from which DNA polymerase synthesizes a single-stranded copy of T-DNA (Wang et al., 1987; Stachel et al., 1986). This single-stranded copy becomes dissociated from the double-stranded plasmid. A protein, encoded in the vir region, binds to the 5' end of the single-stranded T-DNA (Citovsky et al., 1988; Das, 1988; Gietl et al., 1987), and this complex alone apparently enters the plant cell (Caplan et al., 1983). The T-DNA is the only portion of the Ti plasmid that is stably incorporated into recipient plant cells. Having entered the plant nucleus, the T-DNA integrates into the host chromosome, and a complementary DNA strand is synthesized (Yadav et al., 1980). The newly integrated genes then direct the synthesis of substances that alter normal plant metabolism. New auxin and cytokinin biosynthetic pathways, and biosynthetic pathways for opines, are expressed. Constitutive synthesis of phytohormones results in uncontrolled cell division (Akiyoshi et al., 1984; Barry et al., 1984; Schröder et al., 1983) that interferes with plant morphogenesis and cell differentiation, and in tissue culture prevents the formation of whole plants from single cells or from callus tissue. The genes of the central core of the T-region thus can induce and maintain tumors but cannot promote their own transfer between plant cells.

Genes for tumorigenicity, as well as for opine synthesis and degradation, are encoded by the Ti plasmid (Van Larebeke et al., 1974; Watson et al., 1975; Gurley et al., 1979; Garfinkel and Nester, 1980; McPherson et al., 1982). Opines are not produced in normal plant tissues. A. tumefaciens uses the released opines as sources of carbon, nitrogen, and energy. Environments containing opines are uncommon and specialized microbiological niches, so it is likely that few bacteria other than those containing Ti plasmids would be able to benefit from the opines produced by plant tumors (Hardy, 1981).

4.3.2 Disarmed Ti Plasmid

Disarmed Ti plasmids have been constructed from natural Ti plasmids by deleting the tumor-inducing genes from the T-region. Removal of the tumor-inducing genes allows efficient DNA transfer and is essential for plant genetic engineering since these genes interfere with regeneration of normal fertile transgenic plants (Yang et al., 1980; Joos et al., 1983; Leemans et al., 1982). The deletion of the tumor-allows other genes to be inserted by conventional cloning techniques. Along with the 25 bp repeats on the Ti plasmid required for insertion, any additional DNA inserted between them is cotransferred and integrated into the plant nuclear genome (Hernalsteens et al., 1980). At least 14 kilobases of inserted material can be transferred efficiently (Caplan et al., 1983). For this to occur, the 25 bp border sequences must be present on the actual molecules that are to be transferred (Wang et al., 1984). Only DNA sequences located between them are efficiently transferred and
integrated. Deletions or mutations in the left border sequence do not significantly affect transfer, but transfer is totally abolished if the mutations are located in the right border sequence (Joos et al., 1983; Veluthambi et al., 1988). The T-region seems to integrate randomly into chromosomal DNA. Modified Ti plasmid is an efficient vector for transferring DNA inserts into dicotyledonous plant cells, and the resulting transformed plant cells can be regenerated to form fertile plants.

4.3.3 Binary Vector System

DNA Plant Technology utilized a modified "binary vector" system (Hoekema et al., 1983) to transfer genes into excised (explanted) pieces of tomato tissue. The act of tissue excision is analogous to wounding so that the cells therein are then recognized by Agrobacterium as "wounded" targets. The binary plasmid system DNA Plant Technology utilized is derived from the one described by van

den Elzen et al. (1987). This system uses a plasmid that replicates autonomously in E. coli and A. tumefaciens. The plasmid, pJJ2964, harbors the DNA sequences that will ultimately be introduced into the plant. This plasmid was constructed by fusing the fragment from pAGS111 (van den Elzen et al., 1985) that contains the right and left border sequences flanking a NPT II gene cassette into the broad host range plasmid pRK290. The advantage of pRK290 is that it can replicate autonomously in a broad range of bacteria including A. tumefaciens and E. coli. The construct was further modified with a fragment of pBR322 to give a unique Hind III cloning site internal to the border sequences and proximal to a unique Bam H1 site. Two AFF constructs, AFF1 and AFF3 were cloned into the Hind III/ Bam H1 sites to yield pJJ2964-AFF1 and pJJ2964-AFF3.

The two plasmids, pJJ2964-AFF1 and pJJ2964-AFF3, which were initially cloned in E. coli were mobilized into A. tumefaciens with the aid of a helper plasmid. The plasmid pRK2013 (Ditta et al., 1980) contains the genes required for mobilizing desired DNA sequences from E. coli to Agrobacterium. The genes were introduced into the Agrobacterium vector agent from E. coli in a process called triparental mating, which involves mixing the Agrobacterium recipient and each of the two E. coli strains, one carrying pRK2013 and one carrying a pJJ2964-derivative, under appropriate selection conditions. The recipient strain of A. tumefaciens, LBA4404 (Hoekema et al., 1983), contains a Ti plasmid that is completely deleted for the T-DNA region but still retains an intact vir region. Therefore even though it is completely disarmed in terms of its ability to incite tumors, it retains the functions required to mobilize the region between the right and left borders into plants. It is able to mobilize this region in trans, i.e. even if these sequences are contained on a plasmid separate but co-resident in Agrobacterium. Thus, the binary system involves two plasmids: A disarmed Ti plasmid that contains the mobilization functions, vir, and a second plasmid containing the right and left borders on which the vir gene products act to transfer the sequences inserted between the borders into the plant genome.

The modified T-DNA region carrying the genes of interest is then transferred to tomato by cocultivation of leaf disks with Agrobacterium harboring the plasmids (Horsch et al., 1985). The tomato line transformed was L. esculentum var. `Bonny Best'. Transformants were identified by their resistance to the antibiotic kanamycin, conferred by the marker gene NPT II. The presence and expression of the AFF genes were determined by mRNA and protein levels and bioassays.

4.3.4 Promoter, Transcription Termination, and Polyadenylation Signal Sequences

Promoters are regions on a DNA molecule to which RNA polymerase binds and initiates transcription. Promoter DNA sequences are located upstream from structural gene sequences and are not themselves transcribed into mRNA. A key constituent of a useful expression vector is a strong promoter (Tempe and Goldmann, 1982). Such a promoter provides for efficient mRNA synthesis so that high levels of a desired protein will be produced (Willmitzer et al., 1983; Bevan et al., 1983). DNA Plant Technology has utilized two promoters that are constitutively expressed. For expression of the NPT II gene product, DNA Plant Technology has employed the promoter from the nopaline synthase (nos) gene, derived from Ti plasmid (van den Elzen, 1985; An et al., 1985; Herrera-Estrella et al., 1983; Fraley et al., 1983; Koncz et al., 1983; Velten et al., 1984). Expression of the chitinase gene is controlled by the 35S promoter derived from the Cauliflower Mosaic Virus (CaMV). The 35S CaMV promoter sequences have been characterized by Nagy et al., 1985; Odell et al., 1985; and Nagata et al., 1987.

Other key constituents of useful plant expression vectors are DNA sequences that signal that transcription is to be terminated and that the transcribed mRNA is to be polyadenylated. Most eukaryotic mRNAs possess at their 3'-termini a heterogenous tract of between 20 and 200 polymerized adenosine monophosphate residues (called poly A). This "poly A tail" is not encoded in the DNA (i.e., there is no corresponding stretch of thymidine residues at the end of the gene) but is added posttranscriptionally (Mainwaring et al., 1982). The signal sequence for transcription termination and polyadenylation of the chitinase gene was derived from the nopaline synthase gene encoded on the Ti

plasmid of nopaline-metabolizing strains of A. tumefaciens. The corresponding signal sequence for the NPT II gene was derived from the 3' end of the octopine synthase gene encoded on the Ti plasmid of octopine-metabolizing strains of A. tumefaciens.

All DNA fragments integrated into tomato plants using Ti-derived expression vectors have the configuration shown below.

Plant-specific Promoter	___	Gene to be Expressed	____	Polyadenylation Signal

In the AFF3 construct, additional regulatory signals were fused to the 5' end of the gene and sandwiched between the promoter and the structural gene. These sequences code for a signal sequence that direct the protein to be exported into the extracellular space. A signal sequence is located at the 5'-end of a gene and encodes a stretch of 15-30 amino acids at the N-terminus of the protein. This stretch characteristically contains two or three polar residues at the N-terminus and a hydrophobic core of large non-polar amino acid residues such as leucine and phenylalanine. The non-polar amino acids allows the protein to pass through the cell membrane system. A protease embedded in the membrane cleaves the signal sequence once the body of the protein begins to pass through the membrane (Lewin, 1990).
DNA Plant Technology has inserted a signal sequence derived from the tobacco pathogenesis-related protein PR1b (van Loon, 1985). The sequence data submitted by DNA Plant Technology demonstrates that this sequence has the hydrophobic characteristics of a typical signal sequence with its core containing a large percentage of leucine and tryptophan. The PR1b protein is a small molecular protein induced by a variety of developmental and pathological conditions. It belongs to a class of proteins believed to be involved in host defense mechanisms. All members of this class of pathogenesis-related proteins are located in the extracellular matrix.

The new gene and its immediate translation product is as follows:

5'CaMV 35S promoter-signal sequence-AFF-nos3'

Signal sequence (30 amino acids)- Protein A/antifreeze protein

The amino acid residues corresponding to the signal sequence will then be cleaved post-translationally to yield an active enzyme.

4.3.5 Gene Insertion is a One-way Process

The scientific literature supports the view that only the T-region is transferred and integrated into the plant genome (Fraley et al., 1986). The sequence that is integrated includes only genes which are contained between the short, well-characterized segments of the Ti plasmid essential for incorporation into the plant genome. In addition, the border sequences themselves are not precisely transferred during the process of insertion of T-DNA into plant genome. This means that the inserted DNA is no longer a functional T-DNA; i.e., once integrated, it cannot be remobilized into another plant's genome even if acted on again by vir genes (Zambryski et al., 1982). All evidence available since the delineation of T-DNA in 1978, plus the accumulated epidemiology of crown gall disease, indicate that T-DNA transfer into plant cells by Agrobacterium is irreversible.

4.3.6 Stability of Insertion

There is a wealth of data showing that A. tumefaciens T-DNA with or without genes for tumorigenicity becomes integrated into nuclear chromosomal DNA as part of the gene transfer process. (A single report has shown that T-DNA can insert into chloroplast DNA (De Block et al., 1985)). Multiple copies and multiple insertion sites have been observed (Ursic et al., 1983), but any relationship between copy number and expression levels of introduced genes remains tenuous (Nelson et al., 1987). No data exists to suggest that certain integration sites are less stable than others. Of course, any DNA sequence in plant chromosomes bears some degree of instability. This is evidenced in nature and in plant breeding by the phenomena of gene amplification, unequal crossing over, chromosomal nondisjunction, and transposon-induced mutation and reversion. As fully integrated pieces of plant chromosomes, T-DNAs are subject to the same mechanisms that govern gene stability as are other plant genes. Once integrated into plant chromosomes, T-DNA becomes no different than any naturally occurring sequence in terms of its stability or its potential ability to persist in the environment independent of the transformed plant milieu. The term "stable insertion" therefore implies a degree of stability that is similar to that of naturally occurring plant genes. Any slight instability that could be demonstrated would not be a cause for real concern, except that the desired trait might be lost. There is no indication that such an instability could in some way be deleterious to any organism except the transformed plant itself.

The T-DNA is stably transmitted through mitosis and meiosis as an intrinsic part of the plant genome, and becomes a new and novel locus. The chimeric DNA constitutes a new and novel genetic locus in the tomato genome, and is transmitted to progeny via seed. The new markers display dominant phenotypes and are inherited in a Mendelian manner (De Block et al., 1984; Horsch et al., 1984). The regenerated transformed tomato plants are phenotypically normal and fertile, although they may grow somewhat more slowly than unmodified plants. Incorporation of the genes into the plant genome has been confirmed by Southern hybridization. Stable, Mendelian transmission of the traits was demonstrated by following the inheritance of antibiotic resistance in progeny through crosses. Expression of the AFF gene in tissues of transformed plants has been characterized by determining mRNA levels, by measuring enzyme activity, and using biocontrol assays.

V. AFFECTED ENVIRONMENT

The field trial will take place on a small plot on agricultural land in Contra Costa County, California. The site will provide adequate physical security. Site monitoring and management practices that create a nonpropagative environment are expected to provide the necessary degree of both biological and physical containment. These factors are described at more length below.

5.1 Field Plot Design

The objective of the experiment is to produce sufficient quantities of tomato fruit containing the modified AFF gene to conduct laboratory studies on the freeze/thaw properties of such fruit. In addition, data will be collected on the post-harvest susceptibility of the fruit to decay caused by fungal pathogens. Field data are to be collected on the general morphological properties and agronomic traits of the transgenic plants. A small number of plants, 100, are to be released in this experiment. The genotypes, all derivatives of the L. esculentum variety `Bonny Best' to be tested are: 1) 2964, a control transformed with the vector only, 2) 2 independent transformants containing the AFF-1 gene, and 3) 2 independent transformants containing the AFF-3 gene. AFF-1 is expressed intracellularly whereas AFF-3 is expressed extracellularly (see section 4.2.1).

The field design will employ a replicate block arrangement with 2 replications of each genotype. The individual plots consist of 5 plants of a single genotype planted on raised beds 20 feet by 5.5 feet. Individual plots will be separated by a 5 foot alley as will the blocks. The entire experimental field will be surrounded by a buffer of corn that will serve as a windbreak as well as a trap plant and visual barrier. The experimental plot is contained in area 40 feet by 80 feet.

Representative fruit will be harvested at three developmental stages, green, breaker, and red for experimental analysis. The fruit will be evaluated at a range of temperatures from freezing to near freezing. The measurements to be taken include determining electrolyte leakage as a measure of the cellular integrity, instron measurements to determine texture deterioration, and color.

DNA plant Technology has designed many layers of containment into their protocol. Commercial cultivars of tomato such as `Bonny Best' are self-pollinating and therefore cross-pollination is limited. The plants will be located at lease 200 feet from any commercial tomato cultivation, a distance more than sufficient to isolate the experimental tomato and prevent inadvertent mixing of seed or fruit during harvest. Each block will be surrounded by corn plants to serve as a wind-break and visual barrier.

5.2 Field Observation and Monitoring

An APHIS representative will inspect the site at the initiation of the experiment or shortly thereafter to verify information about the test site represented in the request for the permit. The site must pass inspection for the test to be allowed to proceed. Farm and greenhouse employees are on duty 7 days a week and will prevent fauna and unauthorized persons from trespassing onto the experimental plot.

General monitoring will be performed by crop management specialists five times per week for plant morphology, vigor, water status, nutrient status, physiology, flowering, disease, insect infestation and damage from invertebrates and vertebrates.

Monitoring practices that ensure that no transgenic plants will survive after the tests are completed will be described in Section 5.4 below.

5.3 Security of the Test Plot

DNA Plant Technology will take adequate precautions to provide for the physical security of the field test plot. Steps will be taken to prevent

unauthorized persons from trespassing by posting signs and having farm personnel on duty 7 days a week.

The experiment is to be conducted at a research facility for which DNA Plant Technology has a long term lease. The facility is located in an agricultural community. It is sufficiently isolated from the public. The plot is surrounded on all four sides by a 10 foot uncultivated border. On three of the sides, the plot is adjacent to large agricultural fields and on the fourth side, there is an irrigation canal and pumphouse approximately 1000 feet from the experimental plot. Lastly, the view of the site is obstructed by an elevation drop, greenhouse, and surrounding crops.

The only access road to the plot is a gravel road located 225 feet from the plot. The public access road is 0.25 miles from the plot. Finally as part of the experimental protocol, the plot will be monitored daily.

5.4 Final Disposition of Test Plants

Harvested material taken to the laboratory will be destroyed by autoclaving. Material harvested but not used in laboratory experiments will be composted. Any plant material not harvested will be uprooted and allowed to desiccate before being incorporated into the soil. The field will then be disked twice. The field sites will be monitored to ensure that no tomato plants have germinated. Any harvested material in the laboratory not used for analysis will be autoclaved. We feel that these steps are sufficient to guarantee the termination of this experiment and prevent any unplanned release.

VI. ENVIRONMENTAL CONSEQUENCES

The risks associated with the introduction of genetically engineered organisms are the same in kind as those associated with the introduction into the environment of unmodified organisms and organisms modified by other genetic techniques. Also, the assessment of the risks of introducing a genetically engineered organism into the environment should be based on the nature of the organism and the environment into which it is to be introduced. These conclusions were recently affirmed in a report issued by a group of distinguished scientists convened by the Council of the National Academy of Sciences to review the key issues in introduction into the environment of genetically engineered organisms. The report states, "For the determination of ecological risk, the biological properties of the [genetically] engineered organism are paramount." (National Academy of Sciences, 1987).

6.1 Impact on Nontarget Organisms

This section of the EA includes a discussion of impacts on nontarget organisms in the environment with particular attention to those that might be threatened or endangered.

6.1.1 Native Floral Communities

As described in section 4.1.3 and subsections of this EA, unrelated plant species cannot be pollinated by tomato. No weedy species related to tomato have been observed in the areas adjacent to the field test plot. In any case, no means of transmitting the inserted gene(s) to unrelated plant species have been identified in nature. Only in laboratory experiments can this transmittal of genes across genetic barriers be made possible. APHIS concludes that it is highly unlikely that the genetically engineered tomato plants will introduce any of the experimental genes into the gene pool of any local native floral community.

The transformed tomato plants have been tested and shown to be free of infection by A. tumefaciens. The inserted genes are stably incorporated into a tomato chromosome, and no mechanism is known to move them to any sexually incompatible plant. Thus, neither plants in nearby agricultural areas nor any wild plants in the surrounding ecosystem will be affected. Because there is no identifiable direct effect of this field test trial on any wild plants, there is no apparent risk to any threatened or endangered plant species.

6.1.2 Native Faunal Communities

No factor unique to these field tests has been identified that would have an effect on any vertebrates. Beneficial invertebrates, such as honey bees, are not likely to be affected; since flowers will not be present. There is no known data to suggest that either the AFF products or the NPT II gene product is toxic to any animal. No risks to wild animals, vertebrate or invertebrate, can be identified.

6.2 Impact on Existing Agricultural Uses

An attempt has been made to introduce freeze tolerance into plants by a nonconventional method. By preventing freeze damage the new means of post-harvest processing and storage of fruit can be introduced. Furthermore, an extension of this technology to crops which are grown in areas where cold snaps can and do frequently lead to yield loss due to freeze damage may protect the plants from damage.

6.2.1 Alteration in Susceptibility to Disease or Palatability to Insects

There has been no intentional change in these plants to affect their susceptibility to disease-causing organisms or palatability to insects, and there is no reason to believe that these characteristics are different in the transformed and untransformed plants. The only physiological changes in the transformed plants are presumed to be the synthesis of two additional proteins, the fusion protein made up of Protein A and the synthetic antifreeze

gene and the gene product of the marker gene, and these are not expected to have any effect on plant disease organisms or insects.

Bacterial, fungal, and viral diseases are, at times, important in tomato production. There is no reason to believe that the genetic changes introduced into the tomato in the test plots should affect susceptibility to fungal, bacterial, or viral pathogens. If there were any changes in disease susceptibility, the effects should be confined to these plants and the test plot. Some insect pests can be limiting for tomato production. The experimental plot will be monitored frequently to detect any abnormal insect populations or unexpected infestation by plant disease organisms. Approved insecticides or fungicides will be applied if necessary.

Unusual changes in insect populations caused by the modified tomato plants could possibly be ecologically significant only if the experimental tomato were propagated extensively in the environment. This will not be the case. To recapitulate, therefore, any environmental impacts relating to plant pests would be at most insignificant and temporary, and would be limited to the test plot.

6.3 Impact on the Immediate Physical Environment

Due to the nature of the transformed and untransformed tomato plants and the safeguards built into this field test, upon termination of the experiment no tomato plants will survive to cause an effect on the physical environment.

6.4 Impact on Human Health

None of the tomato will be available for human consumption. No new unique toxic substances are encoded by any of the introduced genes, nor do their gene products cause the enzymatic production of dangerous metabolites. No potential impact on people living in the area of the field test, or any other human population, can be identified.

VII. CONCLUSIONS

APHIS concludes that no significant risk of introduction or dissemination of a plant pest, and no significant impact to the quality of the human environment, will result from issuing the permit described in this EA. The factors that were evaluated in reaching this conclusion are again summarized as follows:

1. A synthetic gene that encodes an antifreeze protein fused to Staphylococcus aureus Protein A (Protein A-Antifreeze Fusion protein or AFF) has been inserted into the tomato chromosome. In nature, chromosomal genetic material from plants can only be transferred to other sexually compatible plants by cross-pollination. In this field trial, the introduced genes cannot spread to another plant because the test plot is located at a sufficient distance from any sexually compatible plants with which these experimental tomato plants could cross-pollinate. Therefore, the introduced gene will be prevented from spreading to other plants by cross-pollination.

2. Neither the introduced AFF gene itself, nor its gene product, confers on tomato any plant pest characteristics. Traits that lead to weediness in plants are polygenic traits and cannot be conferred by adding a single gene.

3. The organisms, P. americanus and S. aureus, from which parts of the chimeric gene were derived are not plant pathogens.

4. The vector used to transfer the AFF gene to tomato plants has been evaluated for its use in this specific experiment and does not pose a plant pest risk. The vector, although derived from a DNA sequence with known plant pest potential, has been disarmed; that is, genes that are necessary for producing plant disease have been removed from the vector. The vector has been tested and shown not to be pathogenic to any susceptible plants.

5. The vector agent, the bacterium that was used to deliver the vector DNA and the AFF gene into the plant cells, has been shown to be eliminated and no longer associated with the transformed tomato plants.

6. Horizontal movement of the introduced gene is not known to be possible. The vector acts by delivering and inserting the gene into the tomato genome (i.e., chromosomal DNA). The vector does not survive in the transformed plants. No mechanism that can transfer an inserted gene from a chromosome of a transformed plant to a chromosome of another organism has been shown to exist in nature.

7. The gene product, AFF protein, acts by inhibiting ice crystalization. The only possible phenotypic change in these transgenic plants is a decreased susceptibility to freeze damage. Effects on complex agronomic traits such as yield are not expected.

8. DNA sequences used to regulate expression of the inserted genes in tomato are derived from the plant pest Agrobacterium tumefaciens and the cauliflower mosaic virus. These sequences in themselves, however, encode no proteins, and confer no plant pest related property on the recipient plants.

9. The test is to take place on a small field site, approximately 0.25 acre in size. The site has good security: public access is restricted, a visual barrier of corn plants will border the experimental blocks, and employees are on duty 7 days a week .

10. At the conclusion of the test, all plant material left in the field will be uprooted, allowed to desiccate, and then incorporated into the soil. The site will be monitored for any volunteer plants that may arise.

The test has been designed with safety factors to minimize the possibility of adverse ecological effects. At the conclusion of the experiment, plants will be removed, and the fields monitored for any volunteer plants. Should unanticipated effects arise, the isolation of the test site and the manner of conducting the test indicate that the effects can be readily contained and would have no permanent effect on the environment.

This proposed field test will not have a significant effect on the environment. APHIS has determined that this limited field test will not pose a risk of the introduction or dissemination of a plant pest into the environment.

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