Conference Report

WORKSHOP ON SAFEGUARDS FOR PLANNED INTRODUCTION OF TRANSGENIC TOMATOES

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Scientific Chair

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INTRODUCTION

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) regulates the field testing of transgenic crops. APHIS has overseen field tests with more than two dozen plant species and works closely with the developers of the new varieties and with numerous other interested parties and the public to ensure the continued safety of American agriculture. In the interest of understanding issues pertaining to the safety of field trials and agricultural testing and cultivation of specific transgenic crop varieties, APHIS, beginning in October 1990, convened a number of workshops. Crops that have been considered include oilseed crucifers, wheat and corn, potatoes, and rice.

While testing of new crop varieties at preliminary evaluation stages may involve small test plots and relatively restrictive test conditions, performance evaluations at later stages are often of larger scale and conducted under conditions which closely parallel normal agronomic practice for commercial production of the crop. Confinement of propagules from the transgenic crop is more difficult and can be impracticable under these conditions. Consideration of the consequences of growth of particular transgenic crops during these later stages and when grown commercially has been at the focus of these workshops; the topic will be of increasing importance as new cultivars proceed toward commercialization.

APHIS' consideration of these issues with respect to transgenic tomatoes has proven especially timely. On July 14, 1992, APHIS announced receipt of a petition from Calgene, Inc., requesting a determination from APHIS on the regulatory status of particular tomato varieties, called FLAVR SAVR™ tomatoes, which were genetically engineered to have altered softening properties. Calgene requested that APHIS reach a determination that FLAVR SAVR™ tomatoes have no potential for plant pest risk and should not be regulated articles. (The petition was granted by APHIS on October 19, 1992.)

During the course of considering the Calgene petition, though independent from the petition.itself, APHIS convened the workshop summarized in this report to address safety considerations related to planned introductions of transgenic tomatoes. The workshop was held in conjunction with an international conference on "Molecular Biology of the Tomato: Fundamental Advances and Crop Improvement'. Panelists from industry, academia, foreign governments, and public interest groups considered three major issues: (1) the potential for gene movement from transgenic tomatoes to other plants; (2) environmental consequences of such gene movement; and (3) safeguards to eliminate or minimize such gene movement, where appropriate. The information developed in the workshop was useful to APHIS in its consideration of the petition.

The following report contains the Executive Summary of the discussion that took place at the workshop, as well as short invited papers prepared by each of the panelists.

APHIS wishes to thank all of the participants in the workshop for their able discussions of the topics at hand, and all of the individuals, including the panelists, who have read drafts of this document. We are also particularly grateful to the discussion chair, Dr. Fredrick Bliss, Chairperson, Department of Pomology, University of California at Davis, and to our science writer, Barbara Goldoftas. We would also like to thank Dr. John Yoder and members of the conference organizing committee for their willingness to help host the workshop and rearrange conference scheduling to accomodate these discussions.

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EXECUTIVE SUMMARY

Introduction

The cultivated tomato (*Lycopersicon esculentum*) is widely grown throughout the world and has been studied extensively by researchers and plant breeders. Because of the high consumer demand for the vitamin-rich tomato and extensive information available about its genetics and biology, it offers an excellent and immediate opportunity for cultivar improvement through genetic engineering.

The tomato originated in the Americas. After Europeans colonized the continents in the sixteenth century, they took the plant to Europe. Despite superstitions about the tomato's supposed poisonous properties, it spread throughout the world. Now considered to be the second-largest vegetable crop in the United States (though technically a fruit), the tomato is grown from the tropics to within a few degrees of the Arctic Circle. It has been adapted to a wide variety of soil types and climates, and is cultivated on a small scale by home gardeners and commercially on a large scale. The tomato produces an abundant crop. Under conditions of continual harvesting, yields can be produced of more than 20 tons per acre for fresh market tomatoes, 30 tons per acre for processing tomatoes, and up to 80 tons per acre in greenhouses.

As a common subject of research, the tomato has been carefully studied: its physical properties are well understood and the tomato genome has been extensively mapped. Domestication of the tomato has reduced its genetic variability, but the nine species in *Lycopersicon* can be intercrossed, and over the past 50 years traits such as disease resistance, flavor, color, texture, and shape of the tomato have been modified by traditional breeding techniques, including tissue culture and mutagenesis. Scientists expect that, over the next few years, genetic engineering techniques will enable them to further alter the crop's flavor, color, fiber content, shelf life, and resistance to disease and insects. The USDA has already issued more than 60 permits for field trials using one or more of 20 different transgenic lines.

To examine the potential biosafety issues relevant to the development and commercialization of transgenic tomatoes, the University of California at Davis and the USDA's Animal and Plant Health Inspection Service sponsored the "Workshop on Safeguards for the Planned Introduction of Transgenic Tomatoes" on August 19 and 20, 1992, at the University of California, Davis. It was held in conjunction with an international conference, "Molecular Biology of the Tomato: Fundamental Advances and Crop Improvement". The workshop brought together 15 experts to examine the following issues:

*the potential for gene transfer from transgenic tomatoes to their wild relatives or other organisms

*the environmental consequences of this gene transfer

*safeguards that could be used to minimize or eliminate the possibility of gene transfer.

Because of the regulatory purview of the USDA, the panelists deliberately did not address issues of food safety or economic and ethical considerations.

The workshop, which was chaired by Dr. Fredrick Bliss, Chairperson, Department of Pomology, University of California at Davis, drew an audience of about 40 individuals. There were two sessions, each of which included formal presentations by panel members followed by discussion among the panelists and questions from the audience. Panelists were from academia, industry, the public interest sector, and government.

The discussion focused largely on questions of whether genes could escape from cultivated transgenics to wild plants and other organisms, whether the genetic engineering of new tomato cultivars poses a unique risk compared with traditional breeding methods, how risk assessment should be approached, and what constitutes weediness. The panelists overall agreed that, because of the particular transgenes being introduced at this time: transgenic tomatoes do not present an environmental risk; that the safeguards currently used in conducting field trials and in processing are probably sufficient; and that additional safeguards might only be necessary in the future for particular novel transgenes. Relatively little of the discussion dealt with specific transgenes that might warrant further consideration or regulation, or on particular safeguards or potential regulations that should be adopted.

Part I: Gene Transfer

Like other tomato genes, transgenes in genetically engineered tomatoes potentially could "escape" at various stages through either pollen or seeds: through natural cross-pollination during the growing season; during harvesting; after the harvest, when tomato fruits are left in the field; during transportation to the processing plant, if tomatoes fall from the truck to the roadside; after processing, when the factory waste, including pulp and seeds, is discarded; during seed processing; and in human or animal wastes after tomatoes are eaten.

Much discussion focused on gene transfer through pollen. In order to understand whether or not pollen could be transferred from transgenic plants to other organisms, it is necessary to understand natural cross-pollination in tomatoes. Most past research exploring outcrossing in tomatoes has considered the transfer of pollen from outside the fields into tomato plantings: breeders were concerned about the contamination of their plantings by cross-pollination, and ways that they might exploit natural cross-pollination for hybrid seed production. Nonetheless, this body of research can be useful in assessing the chance that pollen from transgenic tomatoes might escape from an experimental or commercial plot and fertilize other tomatoes.

Dr. Charles Rick of the Department of Vegetable Crops at the University of California at Davis has studied tomatoes for about 45 years. He described factors that influence the rates of natural outcrossing within the genus *Lycopersicon,* and the methods that are used to estimate these rates. Because of natural barriers to pollination, he characterizes the chance that genes might escape through the pollen of transgenic tomatoes as "minimal."

The tomato belongs to the genus *Lycopersicon*, composed of nine closely related species that can all intercross, with varying degrees of difficulty (although not all pairs of reciprocal crosses are possible). Only two *Lycopersicon* species, *L. pimpinellifolium* and *L. cheamanii,* can be easily crossed with *L. esculentum.* The closest relative to *L. esculentum, L. pimpinellifolium,* the currant tomato, grows in Ecuador and Peru. The next closest relative, *L. cheesmanii*, is found only in the Galapagos islands in the Pacific. The wild form of *L. esculentum,* variety *cerasiforme*, is widespread in the Andean region in Central and South America and throughout the tropics in the Old and New World. Thought to be the direct ancestor of the cultivated tomato, it is a weed whose tendency to spread obscures its true range. However, it is not a very pervasive nor an aggressive weed, and is not considered to be a serious weed pest in the United States.

Lycopersicon species are not reported to cross in nature with members of the closest related genus, *Solanum*. Rates of outcrossing within *Lycopersicon* are influenced by natural morphological bamers to pollination and the frequency of appearance of pollinating insects. According to Rick, the cultivated tomato is generally described as a self-pollinating crop. The structure of the flower itself practically guarantees self-fertilization: the anthers are arranged in a tube surrounding the stigma. Pollen drifts down the tube until it reaches the stigma, whose hard-to-reach "depressed" position generally shields it from pollen from other flowers.

The presence of pollinating bees also influences rates of outcrossing. Research shows that in the United States tomatoes are pollinated by bumblebees (*Bombus* spp.) and solitary bees, while in the tropics and subtropics, particularly in the native Andean region, carpenter bees (*Xylocopa* spp.), stingless bees, and others also carry pollen. Dr. Benito Alvarado Rodriguez of the Campbell Soup Company in Mexico pointed out that little information is available about pollination by other insects, particularly in the tropics. While some airborne pollination experiments in greenhouses, in which male-sterile tomato plants were grown among wild relatives within the genus that are prolific producers of pollen, indicated that low rates of hybridization were possible, Rick sees no indication that such pollination is significant in the field.

To estimate rates of outcrossing, researchers test the proportion of hybrid progeny from fertile plants, or, using male-sterile plants as recipients, observe the extent of fruit set and seed production. In either case, they interplant the test plants with normal, fertile plants to compare rates of production of various hybrid genotypes in different seasons and habitats.

Both methods reveal very low rates of outcrossing in the north temperate zone and slightly higher rates in the tropics and subtropics. In the United States, the average rate of outcrossing in a typical field ranges from 0 to 5 percent, with the lower values predominating. (Most often, pollination rates of 0 to 2 percent are observed.) In the Andes, where wild species are more likely to cross-pollinate and where some wild species are self-incompatible, outcrossing rates into *L. esculentum* determined using the male sterile method tend to be about 10 times as high, while rates of detection of hybrid progeny are 30 times higher. Tests on genotypes with a stigma that is greatly depressed within the anther tube, and therefore unlikely to receive outside pollen, show significantly lower rates of outcrossing, barely a fraction of 1 percent.

The proximity of plants is also important. Experiments have shown that as the distance between plants increases, the rate of hybridization drops rapidly. In one set of tests, the rate of cross-pollination was 1.5 percent at 6 feet, 0.7 percent at 18 feet, and 0.2 percent at 30 feet. Beyond 30 feet there was negligible cross-pollination.

Although these experiments investigated the possibility that pollen from outside a test field might cause the production of off-types within the test field, there is little direct experimental data about how pollen from plants in an experimental or commercial field might spread to related species growing nearby. In the United States, said Rick, because there are no other species with which the tomato might hybridize, "outcrossing to related species that might be growing in the vicinity of tomato plantings is an hypothetical situation." With other wild relatives, said Rick, "the migration of pollen from the cultivated tomato to the wild species is blocked. That's just a fact of nature." This blockage arises from the inability of *L. esculentum* to function successfully as the male parent in these crosses. Any introgression of genes would be more likely to go from the wild to the cultivated tomato, rather than the other way around.

Another concern raised related to international aspects of seed production and use. Hybrid seed production, often of seed still being tested in grower trials in this country, frequently occurs in other parts of the world, such as Asia and Central and South America, where more weedy varieties grow and where more outcrossing occurs. Cross-pollination has not been measured precisely in these regions, and a number of participants expressed concern about the possibility that gene transfer there might pose a different level of potential risk.

Similarly, panelists discussed the implications of normal movement of tomato seeds. They recognized that despite restrictions that could be placed on contained field trials or commercial production, the transfer of genetic material could take place through seeds of the transgenic tomato. Once a transgenic tomato is placed on the market, its seed will be dispersed by farmers, gardeners, and consumers during cultivation, transportation, processing, disposal, and even during treatment of plant waste and sewage.

Panelists also considered whether genetic material from a transgenic plant could migrate to organisms that are not plants, such as microorganisms. This movement, termed horizontal gene transfer, has not been well researched. Dr. Nevin Young, Department of Plant Pathology, University of Minnesota, discussed the possibility that an introduced gene might be transferred to a plant pathogenic bacterium infecting the plant. "There's not much risk, but there's not much information either," said Young. He emphasized that this is not a question limited to tomato, but can be applied to any transgenic plant species. Dr. Keith Redenbaugh, of Calgene, Inc., stated that even if it was assumed that gene transfer from tomatoes would always occur, the number of recombinant microbes produced through horizontal gene movement would represent no more than 10^{-10} percent of the microbial population already present. Panelists concluded that experiments could be designed to see if horizontal gene transfer can be detected, to better understand potential impacts, if any.

The panel also came to the consensus that, given the low probability of gene transfer, risk assessment should be done on a gene-by-gene or trait-by-trait basis, rather than solely because a plant is a transgenic.

Summary Section I: Gene Transfer

**Lycopersicon esculentum* is isolated biologically and wild relatives are isolated geographically. The species tends to self-pollinate, there is little outcrossing, and there are few other taxa in the United States that could form hybrids were pollen received from the transgenic tomato.

*Genes could be transferred from one tomato variety, including a transgenic, to another, but the probability of such a transfer is low. Nonetheless, risk assessment should be done on a gene-by-gene basis in the context of existing biological, physical, and geographical factors.

*Introduced tomato varieties can spread from a cultivated field through their seed, although there are no known examples of the spread of specific genes that might pose an environmental threat.

*The ample experience with traditional breeding in tomatoes shows that there are few instances in which plant species that are potential recipients of genetic information from transgenic tomato have undergone any recognizable modification as a result of natural gene transfer from conventionally bred tomato.

*The transfer of genes to microbes or other organisms does not appear to be an issue with the tomato, but because there is so little direct data available for any plant species, some panelists recommended that research in this area should be pursued.

Part II: Probable Consequences of *Gene* Transfer

According to Dr. Jay Scott of the Gulf Coast Research and Education Center, in Bradenton, Florida, conventional risk assessment looks at risk (r) as the product of the probability that a hazard (h) will exist *times* the probability of exposure (e) [$r = h*e$]. In evaluating the probable consequences of gene transfer from transgenic tomatoes to other plants, risk equals the probability that a gene will involve a hazard times the probability that the gene will be transferred to other organisms where it might cause harm. Although the probability that a transgene will *escape* is low, it nonetheless is not equal to zero. Therefore, said Scott, it is important to explore the possible consequences of gene transfer.

Much discussion focused on what would constitute a hazard, and how likely the occurrence of that hazard was. Issues discussed included the potential for transgenes to alter the weediness of the tomato and its wild relatives or to affect insect populations; and the effects of gene transfer occurring in other countries where relatives of the cultivated tomato grow. The possible effects on biodiversity were also mentioned as potential hazards to be considered.

A number of panelists pointed out that although the *escape* of transformed genes seemed unlikely in the United States, if it did occur the potential impact would depend on the particular genes that were involved. Genes likely to be introduced into the tomato are those that affect male sterility, fruit composition and ripening, and resistance to herbicides, insects, and pathogens. Male sterility is an evolutionary disadvantage, and male-sterile plants would obviously be less fit in terms of dispersal than male-fertile plants. Similarly, changes in fruit quality were not seen as a potential threat in terms of weediness. The workshop did not come to a consensus *on* whether conferring insect resistance to transgenic tomatoes might eventually involve a potential hazard.

The possibility of risks in international settings was of particular concern to some panelists. A number of speakers stressed the importance of exploring the question of where *seed* is produced. If transgenic tomatoes were grown in the Andean region, where more outcrossing occurs, there might be a potential for considerable ecological effect. There was agreement that the scenario of an escaped *transgene* altering another species and changing the biodiversity of an ecosystem should be considered, although it was unlikely, particularly in light of the history of conventional breeding.

A concern commonly voiced in consideration of transgenic plants including tomato is the possibility that they might become weed pests or enhance the weediness of a weedy relative. A weed is broadly defined as a plant that grows where it is not wanted, whether among crops or elsewhere. Weedy plants typically are persistent and aggressive. Specific traits that enable them to colonize new areas include hardy seeds, rapid vegetative growth, a short life cycle, and high seed production and dispersal. Although it is well-known that introduced or exotic species can become pests, exotics are generally not considered to be useful models for introduction of transgenic varieties of crop plants.

Tomatoes, though not weed pests, do have some weedy properties. Cultivated tomatoes turn up in fields where they were grown the previous season, i.e., as 'volunteers', even in areas of the United States that are subject to freezing. *L. esculentum* var. *cerasiforme* is a common weed in some parts of the world, but not in the United States; native *Lycopersicon* species are common weeds in the Andean region. The enhancement of specific traits, such as the competitive growth habit, seed dormancy, pathogen or insect resistance, allelopathy, and tolerance to drought or other environmental stresses, conceivably could increase the fitness of the tomato in some situations. However, while tomatoes can establish themselves in new territories, they are not aggressive or persistent weeds, and Dr. Joseph DeVerna of Campbell Soup Company, Davis, California, described the cultivated tomato as having little potential to become a weed pest.

Panelists repeatedly stressed the importance of approaching the possible risks of the transgenic tomato within the context of the results of the past 50 years of conventional breeding in tomatoes. In the discussion of specific hazards, such as weediness or the effects on biodiversity, it was noted that although traditional breeding can cause large and sometimes unexpected genetic changes, past unrestricted experiments, breeding trials, and commercial cultivation have not yielded examples where such hazards were evident. Despite the vast amount of breeding in tomatoes and the great genetic changes, for example, several panelists noted that there has been no reported incidence of weediness. Panelists stressed that, overall, genes introduced through genetic engineering are probably not more dangerous than those introduced via conventional breeding.

An additional concern mentioned was the possibility that the use of plants modified to be insect resistant might create secondary effects on insect populations. For example, Dr. Mark Lagrimini, of the Department of Horticulture, Ohio State University, suggested that the use of natural plant defensive genes conceivably might put such selective pressure on certain insects to become insensitive to these natural defense mechanisms. Dr. Alvarado Rodriguez suggested that further research is needed to look at possible effects that transgenics might have on natural enemies of insect pests. Dr. David Bayer, of the Department of Botany, University of California at Davis, offered the opinion that genes leading to the production of allelopathic compounds should not be introduced into tomato, as they might affect the biodiversity of plant communities.

A further concern emerged from these discussions: while specific traits are unlikely to pose a risk, the introduction of these traits to a wide range of plants might be problematic. For example, several panelists referred to ongoing public dialogues, not unique to agriculture, of the possible adverse consequences of the overuse of a beneficial technology, such as the use of Bacillus *thuringiensis* delta-endotoxin genes in a variety of different transgenic crops. Lagrimini said that the environmental consequences of resistance to *Bacillus thuringiensis* toxin would depend on the regional use of the toxin, its mode of application (i.e., topically or contained in genetically engineered plants), and the role of indigenous *Bacillus thuringiensis* in the soil.

Summary Section II: Consequences

*Classical risk assessment evaluates risk as the product of the probability of hazard and the probability of exposure [r = h*e].

*Environmental risk assessment should focus on individual transgenes and how they affect the tomato and its relatives.

*Certain countries, such as those in the Andean region, with large indigenous populations of relatives of the cultivated tomato, would be more likely sites for adverse consequences, if any. Although conventionally bred cultivars have been introduced in these regions, there have been few examples of crossing with the native populations. Rick stated that he has never seen evidence that a significant trait of the domesticated tomato, a determinative habit, has been introgressed into wild populations of the closest relative, *L. esculentum* var. *cerasiformae*, even when crop varieties and wild populations are near one another. However, local studies of gene transfer and its possible effects should be conducted if these novel tomato varieties are introduced there.

*The genes that pose the most concern are those with the potential to change weedy characteristics of the tomato or its wild relatives. Although *Lycopersicon* species tend not to be weeds, introduced genes that have the potential to enhance aggressiveness should be considered on a case-by-case basis.

*Specific traits likely to be introduced in the transgenic tomato seem to involve little risk, particularly in light of the long history of tomato breeding.

*Possible concerns from the overuse of particular beneficial genes in a wide range of plants and potential secondary effects on populations of insects and other organisms, bear consideration.

Part III: Safeguards

The history of conventional plant breeding and the more recent experience with field trials of transgenic tomatoes in the United States give useful information about effective safeguards that should be adopted to contain transgenics during field trials.

Dr. Ben George of Heinz U.S.A., Tracy, California, described the "fairly rigorous testing procedure" that most companies use in determining whether a new crop variety is acceptable. After research trials in several seasons indicate that a new cultivar is worth pursuing, it is tested in industrial trials, which begin with small grower strip trials on plots of 1 to 2 acres the first year and then expand to 5- to 10-acre experimental trials the second year. Overall, a new cultivar is probably tested for three or four years before it can be sold commercially.

Panelists agreed that the current safeguards for conducting contained field trials of tomatoes, transgenic or otherwise, are sufficient. Dr. Keith Redenbaugh of Calgene, Inc., provided a comprehensive overview of the basic physical safeguards he believed to be adequate during field trials of transgenic tomato, during and after harvesting, and during processing.

First, there should be at least a 30-foot isolation zone to separate the trial plot from other tomato fields and thereby prevent outcrossing. This isolation zone can be kept barren or planted with a tomato border or another crop. Second, all equipment used in the test site should be cleaned on site and the plant debris should be left there. Third, harvesting should be conducted separately from that of any adjacent tomato fields. Similarly, seed processing operations should be conducted separately from other processing. Finally, after harvest the field should be disked, watered, and monitored for six months in order to control volunteer plants. Dr. Sheila McCormick of the Plant Gene Expression Center, Albany, California, also suggested that it should be standard procedure to rotate crops in the trial sites.

A number of panelists mentioned that while the spread of pollen from transgenic tomatoes to other plants should be minimal, the ability to control pollen movement from commercial fields is limited. Panelists agreed that it would be impractical to require such practices as emasculating or deflowering plants or keeping pollinators away by bagging inflorescences or using nets and traps. However, McCormick suggested that a reporter gene could be included to assess the spread of a transgene into border rows of non-transgenic tomatoes.

In addition to physical safeguards, temporal safeguards were seen as useful in containing transgenic plants. For example, in field tests transgenics could be planted at a different time than commercial tomato plantings or gardens so the plants do not flower simultaneously.

Louise Duke of Agriculture Canada reported similar safeguards in Canada, where trials with transgenics have been conducted since 1988. For the transgenic tomato, the minimal distance required to provide an isolation zone is 100 meters, or about 328 feet. After harvest, the land cannot be planted with the same or closely related species for one year.

While panelists agreed that safeguards for commercial production should be based on the risk identified for a particular transgene and the probability that it might pose a hazard, they also pointed to the difficulty of regulating widespread use of a particular transgene in many crops. Current practices for tomato production and processing are seen as providing certain safeguards against gene transfer and potential environmental consequences. Safeguards that attempt to change farmers' practices or target consumers were seen as generally impractical.

Summary Section III: Safeguards

*Current safeguards for conducting contained field trials of the transgenic tomato are sufficient.

*If any special safeguards for commercial production of transgenic tomatoes are deemed necessary, these should be established on a case-bycase basis according to the risk identified for a particular transgene and the probability that it may pose a hazard.

*Established practices for tomato production and processing provide a considerable measure of safety against gene transfer and potential environmental consequences.

INVITED PAPERS

Paper 1

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Introduction

Previous field studies conducted in Sinaloa, Mexico, have indicated that tomato transgenic lines with a *B.t.* gene offer an effective alternative for controlling two of the most important key pests in the area, the tomato pinworm *Keiferia lycopersicella* and the tomato fruitworm, *Helicoverpa zea* (Boddie) and *Heliothis virescens* (Fabricius). However, before these kinds of tomatoes can be commercially utilized, their potential impact in agroecosystems needs to be analyzed. I would like to express my opinion based on experience I have had with *B.t.* tomatoes in the following three general areas: gene transfer; environmental consequences of gene transfer; and safeguards.

I. Gene transfer

Gene transfer from tomato transgenic lines to neighborhood commercial processing tomato varieties via pollen is unlikely to occur due to reproductive morphological barriers present in tomato flowers. However, to my knowledge specific studies specifically addressing this aspect have not been conducted yet. Different species of pollen feeding insects, wind, and birds are potential pollen vectors that should be considered in such type of studies.

The gene transfer issue acquires greater importance in areas that are centers of diversity where wild tomatoes and weedy relatives occur more abundantly all year round. Therefore, above proposed studies are of prime importance in these geographical areas if genetically modified tomatoes are intended to be commercialized.

II. Environmental consequences of gene transfer

If *B.t.* gene transfer occurs from tomato transgenic lines to commercial tomato varieties, wild tomatoes or weedy relatives, several negative ecological consequences might be expected and among any others, I would mention the following:

(1) Natural enemies of key insect pests in different agroecosystems are most important regulatory factors that need to be protected to ensure their survival and utility. This is of particular importance for sub-tropical regions in the world. So far, no specific studies have been conducted to determine the impact of *B.t.* modified tomatoes on the natural fauna.

(2) Another potential ecological impact is the promotion of development of insect pests resistant to introduced genes such as the *B.t.* gene, particularly if gene transfer occurs to non-intended tomato plants. Management strategies based on research studies have to be individually designed, considering each specific area where *B.t.* modified tomatoes are planned to be utilized, in order to delay as much as possible, the development of resistance to *B.t.* genes that sooner or later would occur.

Management strategies derived from research studies have to be designed *a priori* by considering each specific area where *B.t.* modified tomatoes are planned to be utilized, in order to delay as much as possible the development of resistance to *B.t.* genes that sooner or later would occur.

III. safeguards

A. Physical safeguards

Acceptable isolation distances cannot be established until factors involved in gene transfer are determined for each agroecosystem for world tomato production.

Emasculation, deflowering, bagging of inflorescence, use of any kind of wall, nets, and traps require a great deal of effort and time and, therefore, are of little practical value in the conduct of transgenic tomato trials. I should add that all the above proposed physical safeguards need to be evaluated for each agroecosystem before any could be implemented as general rules.

B. Temporal safeguards

Planting dates offer a good alternative to mitigate gene flow out of transgenic tomatoes, as the use of appropriate planting dates could avoid flowering synchrony with other commercial tomato plantings.

C. Biological safeguards

Male sterility offers a unique system with great potential to mitigate gene flow. This technique could be useful as part of resistance management scheme for insect key pests that commonly occur on tomatoes. Again, before the male sterile technique could be implemented as a biological safeguard and as part of resistance management, it is necessary to obtain technical information about the properties of these tomato lines in research studies specifically designed for those Purposes.

Paper 2

SAFEGUARDS FOR PLANNED INTRODUCTIONS OF TRANSGENIC TOMATOES: WEEDS

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To assess the risk of a transgenic organism (tomato) released into the environment one must be able to determine the probability that the transgenic organism or genes will cause a problem as well as the probability that the organism or genes will move from the planned site to where they may cause a problem. One of the major concerns from releasing transgenic plants is the potential of creating new serious weeds by adding aggressivity to existing plants and the potential impact on genetic diversity. I will restrict most of my comments to the potential problem of weeds or enhanced aggressivity produced by transgenic plants. Because the term "weed" means different things to different people, I will define a weed for purposes of this discussion as plants growing spontaneously in ecosystems and are deemed to have negative impacts by a segment of society.

It may be possible to establish that certain traits, i.e. changing growth habit, maturity date, enhanced flavor, etc. will not contribute to weediness in known environments and at least theoretically would not contribute to aggressiveness of any species regardless of the situation. This statement must be taken cautiously, however, as these traits or genes may enhance the fitness of the plant under a given environment and allow it to take on a different dimension in the plant community. Other traits, i.e. enhanced competitiveness, improved seed dispersal, seed dormancy, drought resistance, enhanced cold tolerance, allelopathy, etc., may contribute more directly to weediness. Ideally, research should produce crops that are under predictable control and will remain limited to the field in which they are planted.

The genus *Lycopersicon* is closely related to *Solanum*, a genus with a number of weedy species. Whether hybridization will occur between the transgenic plant and the weedy species requires detailed information on compatibility between the various species involved as well as location and distribution of related species. Introduction or spread of a transgenic plant can follow one of three patterns: (1) a short period of persistence followed by eventual disappearance; (2) establishment of one or more stable populations; (3) continued spread and growth until constrained by a limiting factor such as temperature, growing season, moisture, light, space, etc. Whether populations of the transgenic tomato will act as weeds can be assessed early on because the plants can become volunteers in the field in which they were planted. Tomato seed frequently germinate in the field the year following a tomato crop and any transgene that would enhance the aggressivity of these tomato plants should be considered very seriously. A more difficult problem would be if the transgenic plants formed ruderal populations that could survive and create problems later or invade into surrounding plant communities. This latter problem is harder to assess.

Hybridization with other plants in which the transgene will enhance its fitness or aggressivity could be very serious. Not all members of *Solanum* will hybridize with *Lycopersicon* nor are all members of the genus *Lycopersicon* genetically compatible with each other. Even if they were, they must hybridize during the growing conditions of the transgenic crop. However, the "rare" cross represents a serious concern and should be viewed with caution not only as a potential crop weed but as a ruderal in non-agricultural situations.

It has been stated (Keeler, 1990) "that many of the genes that can effectively be transferred to plants and that show economic potential are genes which confer biotic resistance, that is, resistance to other organisms including herbivores or diseases". Biotic interactions can impact plant numbers, size, etc. If these interactions keep a plant small and rare, the transgene will seemingly not be a problem; however, if the transgene alters these biotic interactions and the plant acquires increased biotic resistance, it will concomitantly increase its fitness and may thereby have the potential to become more aggressive. This potential for altering biotic interactions using transgenes that could influence plant aggressivity needs to be taken very seriously.

An obvious issue is the need to compile information concerning remote possibilities even under adverse conditions. This can be extremely challenging because of the absence of a documented history. If public interest groups suspect that a transgenic plant or a plant containing a transgene could possibly develop, through mutation or whatever, into a major weed, they would be remiss not to recommend against its release. The introduction of transgenic plants is not unlike the introduction of exotic plants in which the exotic plant may place new stresses on the ecosystem. There have been many cases where species have been intentionally introduced for a useful purpose and ended up creating enormous problems. We can benefit from the pesticide debate where insufficient research was conducted in the more obscure areas to clearly protect the public and the environment.

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Paper 3

GERMINATION AND DORMANCY OF TOMATO SEEDS

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The dissemination and persistence of seeds from a transgenic crop is an important consideration in determining the potential for escape of the germplasm from the intended planted *areas.* This contribution will review the germination and dormancy characteristics of tomato seeds in relation to this question.

Seed germination and dormancy characteristics contribute to the potential for germplasm spread and weediness. Most weedy species have some type of seed dormancy mechanism to ensure that all seeds do not germinate simultaneously, leaving a seed bank in the soil for the establishment of plants in future years. Species lacking seed dormancy are unlikely to become weedy, as the seeds will germinate readily, even when conditions are not conducive to survival of the resulting plant. Future establishment of plants is also dependent upon regrowth and fruiting of parent plants each year, making elimination of the undesirable plants much easier than eliminating a large dormant seed bank from the soil.

Seed dormancy is largely absent from cultivated tomatoes. Most cultivars exhibit little or no postharvest dormancy period, being able to germinate any time after removal from the fruit when given suitable moisture (water potential greater than –1 MPa) and temperature (8-10°C minimum, 35-38°C maximum) conditions. In some cultivars, the level of dormancy is so low that there may even be a tendency for seeds to germinate within the fruit if they become overripe. Mature seeds generally do not require light for germination, although a red light requirement can be demonstrated in seeds after pretreatment with far-red light. Postharvest seed dormancy that is present in cultivated tomatoes is generally of the type that is removed by a short period (up to a few months) of dry after-ripening or moist chilling. These conditions would generally be met in the field during the fall or winter, allowing most seeds to germinate the following year. General observations indicate that cherry tomato genotypes (var. *cerasiforme*) may have a greater tendency for seed survival in the soil and reestablishment of plants in subsequent years than larger-fruited cultivars.

The situation with respect to seed dormancy is somewhat different in the wild tomato species, which often exhibit postharvest dormancy. Dormancy in the wild tomato species seem to be associated with the seed coat or the endospexm cell layers enclosing the embryo, as their removal or weakening by, e.g., hypochlorite treatments, is generally sufficient to break dormancy and promote germination. Germination in the soil would be similarly delayed until natural forces and microbial action had weakened the seed coat or endosperm sufficiently to allow radicle emergence. Essentially all types of tomato seeds will germinate readily if the seed coat and endospexm cap covering the embryo are removed, indicating an absence of embryo dormancy.

Given these considerations of seed germination and dormancy characteristics, the potential for transgenic tomatoes to become persistent or aggressive weeds outside of the intended cultivated area by dissemination of seed is quite low. Seeds can be readily carried by birds or mammals or washed from fields by irrigation, but it is unlikely that these seeds would establish persistent plant communities outside of cultivation due to the absence of seed dormancy. The dormancy present in wild species is not a serious consideration, since wild species are the source, not the target, of transgenes. It is possible that the seed dormancy trait could be transferred to cultivated lines in association with a desired trait, but this is highly unlikely. Selection and breeding programs that would inevitably follow the initial gene transfer (whether by sexual or molecular means) virtually always involve rapid passage through generations by replanting seed soon after harvest. Seeds exhibiting strong dormancy would be strongly selected against in such a program, so the likelihood of retention of deep seed dormancy in a released tomato cultivar is very low. Lines that did not give seed germinating a minimum of 90% without any dormancy-breaking treatment would be unlikely to be developed or released by the seed industry.

The precautions to prevent contamination of seed stocks in subsequent years by plants from a transgenic crop grown in a prior year would be similar to those currently taken to maintain the genetic purity of current cultivars (George, 1985). Tomatoes for seed should not be grown on fields previously planted to tomatoes within the past two years (minimum). Herbicides and cultivation can eliminate any volunteer plants prior to fruiting within this time period, and soil fumigation with a chemical sterilant is also effective in killing remaining seeds. Due to the high value of F, hybrids and the investment in developing a transgenic tomato cultivar, it is likely that only these hybrids, rather than open pollinated (pure line) cultivars, will be released from a transgenic breeding program. The stock seeds of parent lines are produced under close supervision of the seed company, F, hybrid seeds are produced by hand-pollination, and the fruits are individually tagged and harvested. The opportunities for accidental contamination by or release of transgenic seeds in other seed stocks available to the public is therefore exceedingly low.

Seed present in harvested material is a possible source of escape, as viable seeds will be present in all fresh tomatoes. Home gardeners, for example, could save seed from purchased transgenic tomatoes for their own use. Since F_1 hybrid seeds will segregate in subsequent generations, the products from such saved seed may be inferior to the original and thus not be maintained subsequently, but it would be possible for dedicated amateurs to propagate plants containing a transgene in this way. Seeds discarded in household garbage would be viable and could establish plants near dumps, but for the reasons discussed previously are unlikely to contribute to weediness or transfer transgenes to cultivated lines. Similarly, seeds are a by-product of the tomato canning industry. Processing waste also contains seeds, but the initial stages of extraction of the juice generally involve heating the crushed fruits to increase recovery of solids. The heat treatments employed during processing of the pulp would be lethal to the seeds.

Overall, the seed germination and dormancy characteristics of cultivated tomato seeds are those of a highly domesticated species dependent upon human cultivation for their preservation and maintenance and are unlikely to contribute to weediness as a result of gene transfer to modify agronomic or marketable quality traits.

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Paper 4

ENVIRONMENTAL RISKS OF TRANSGENIC TOMATOES

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The United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS) has received a petition to exempt from regulatory oversight certain transgenic tomatoes that contain antisense polygalacturonase constructs. If the exemption is granted, these transgenic tomatoes could be used in commercial production over large areas of land for many successive years, perhaps indefinitely. Although small-scale field tests can be carefully supervised and monitored, we cannot expect farmers to do the same. Thus, before commercial use is allowed, we must be satisfied either that escape into the environment cannot occur, or that escape will have no environmental effects.

We should not depend on safeguard procedures to prevent escape of transgenic plants or transgenes themselves. Farmers will be reluctant to implement safeguard procedures unless these procedures are already part of their standard practice, or unless there are provisions to enforce compliance with the safeguard procedures. Even if one or both of these conditions can be met, however, lapses will certainly occur that may lead to escape.

One possible exception is safeguards built into the plant that require no new action by the farmer. An obvious example is male sterility. Another possibility is the use of genetic contructs that in principle make escape impossible through some means. The stability of such constructs must be closely examined to insure that the safeguard cannot be breached. Genetic events like mutation, coupled with recombination and natural selection, could render these safeguards ineffective.

Environmental introductions of transgenic organisms have been frequently compared to introductions of non-indigenous species. This comparison is controversial, but we may make valid inferences concerning the effects of scale on introductions. Generally, introductions of non-indigenous species are more likely to be successful if the scale of the introduction is large (that is, if many individuals are introduced; or if the introduction is repeated over many years; or if the introduction is over a large area) than if the scale is small. There is no reason to assume that this principle will not be applicable to introductions of transgenic organisms.

Large-scale commercial use of transgenic tomatoes could lead to escape of the transgenic plant or the transgene itself, with the result that a persistent plant population containing the transgene could become established. Three questions need to be addressed before an exemption is granted: (1) To what extent can we make inferences or extrapolations from small-scale field tests to large-scale commercial use of the transgenic plants? (2) Can we assume that small-scale field tests are sensitive enough to detect rare genetic events that might lead to escape? (3) Can we assume that the risk of escape and establishment increases linearly with the scale of the introduction?

It seems unlikely that transgenic tomato cultivars, *Lycopersicon esculentum*, themselves would become weeds. It seems more likely that pollen poses a greater risk of gene transfer or escape. Tomato cultivars are considered to be strictly self-pollinating due to their short styles. These short styles apparently developed due to inadvertent selection under cultivation. It insures that the stigma is pollinated by the anthers of the same flower. This morphology, however would not prevent pollen from escaping and pollinating flowers of the weedy and outcrossing var. *cerasiforme*, which presumably has long styles.

Variety *cerasiforme* exists in south Texas and south Florida. Consequently, it is a potential recipient of pollen from transgenic tomatoes in those regions. Current information on outcrossing depends mostly on data from tomato cultivars, which are self-pollinators. This may not be the appropriate database from which to make inferences concerning outcrossing from tomato cultivars to var. *cerasiforme*. Standard isolation distances for production of quality seed may be completely irrelevant to the issue at hand. These distances are designed to prevent pollen from coming into a population that is self-pollinating, rather than to prevent pollen from escaping to an outcrossing population.

One issue that has not received much attention is the role of humans as agents of seed dispersal. I once had a summer job mowing lawns around a sewage treatment facility. Periodically, sludge, derived from human wastes, was turned out into open beds to dry. We called these beds "the tomato patch" because they were covered with tomato seedlings. Apparently, seeds from tomatoes eaten raw passed intact through both humans and the facility. This observation indicates that to prevent escape it may not be sufficient to control pollen or seeds on the farm. In addition, although volunteers such as these may not pose a major weed threat, they could serve as sources of pollen far removed from farms and tomato production areas.

The potential for environmental effects depends to some extent on the nature of the transgene. Transgenes that confer or affect some ecologically important phenotype pose greater environmental risks than those that do not. Transgenes that confer insect or virus resistance to the tomato clearly have ecological implications. Such transgenes could improve the fitness of plants, such as variety *cerasiforme*, that receive them. These assertions of course assume that the transgenes will not have pleiotropic effects. Because pleiotropic effects are usually unexpected, they are difficult to anticipate and measure.

A nagging question that remains unanswered is the potential for superinfecting viruses to recombine with viral coat protein genes in transgenic plants, or to become transencapsidated by the foreign coat protein. An effectively new virus could result, with different host range or pathogenicity than the parental virus. Little data is available to help evaluate this question. Until data is available, assertions concerning the safety of these transgenic tomatoes should be considered hypotheses that must be tested experimentally.

Paper 5

GENETIC VARIABILITY IN THE TOMATO

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Introduction

The deliberate introduction of plant species into non-native environments has on occasion resulted in some of the introduced species becoming pests (Pimentel *et al.*, 1989). This is a serious issue and we must leam from these experiences to prevent recurrences.

It is clear that the ability of a species to behave aggressively as a pest will decrease as the species is genetically modified to suit the needs of man - especially in the case of highly-bred row crop such as the tomato. In fact, many domesticated plants and animals cannot survive in nature without the help of humans, and cannot even compete with their wild relatives (Stebbins, 1988). An examination of tomato variability, genetics and breeding will shed some light on the likelihood of a genetically modified tomato becoming a pest.

Tomato genetic variability

In comparison to many other crop plants, the tomato is not highly variable. As a result, early in this century geneticists interested in chromosome mapping and other genetic studies had to rely on a small number of variants identified in primitive cultivars or occurring spontaneously (Stevens and Rick, 1986). Later, they used physical and chemical mutagenesis to supplement this variation (DeVerna and Paterson, 1991). To this date, over 650 morphological and isozyme mutants have been identified; few of these variants have commercial value, and none increases the ability of tomato to behave as a pest. In fact, most of the mutants adversely affect plant vigor and performance.

In contrast to artificially derived monogenic mutants, the wild *Lycopersicon* species possess many of the features typically associated with aggressive plant species (most notably indeterminate growth habit, small fruit size and seed dormancy). Tomato breeders have depended heavily on the wild species as valued sources of monogenic disease resistance (Rick *et al.*, 1987). In contrast. for quantitative variation, selection among modem lines has been of premier importance. In recent times, however, the wild species have become increasingly important as a source of quantitative variation, especially for fruit quality features. Despite the aggressive potential of the wild *Lycopersicon* species, and their extensive use in tomato breeding, no significant "pest episodes" have been reported.

Genomic changes through genetic modification

Recent advances with the use of DNA probes (genomic or cDNAs) have made it possible to evaluate the effects of gene introgression in a way previously not possible. With tomato, there have been several recent examinations of "linkage drag" resulting from the introgression of monogenic disease resistance. The extra chromosomal material introgressed from the wild species has some bearing on genetic modification and the development of pests.

In the case of tobacco mosaic virus resistance (*Tm-2*) derived from *Lycopersicon peruvianum*, Young and Tanksley (1989) found that the size of the introgressed chromosome segment varied from four to fifty-one cM. This was observed despite the fact that as many as twenty-one backcrosses were carried out. The extent of "linkage drag" clearly defied expectation and is likely a result, at least in part, of reduced homology between the wild and domesticated species.

Another example is root knot nematode resistance (Mi), a trait also derived from *L. peruvianum*. Modem tomato cultivars and hybrids surveyed by Messeguer *et al.* (1991) were found to possess from less than two cM to up to 8cM of the *L. peruvinaum* chromosome.

The *Tm-2* and the Mi examples are relevant to the pest topic in two ways. First, despite the pest characteristics of the *Tm-2* and Mi donor, none of the derived disease-resistant cultivars show any enhanced pest tendency. Second, the size of the introgressed segment is enormous in comparison to that expected using non-traditional genetic modification. One cM in tomato approximates '500 kilobases (Messeguer *et al.*, 1991). Thus for *Tm-2* and *Mi* the range of the inserted chromosome segment varies from 2,000 to 25,500, and 1,000 - 4,300 kb, respectively. In addition to the size of the introgressed segment, it is likely that differences in chromosome structure and homology occur between the tomato and its wild relatives. Evidence for non-homology comes from examination of chromosome pairing during pachytene in interspecies hybrids (Khush and Rick, 1963) and from a restriction fragment length polymorphism (RFLP) examination of the *Lycopersicon* species (Miller and Tanksley, 1990).

Gene transfer using non-traditional genetic modification is much more specific than with classical breeding. For example, with Calgene's FLAVR SAVR™ tomato, a single insertion event adds only 7.850 kb DNA to the tomato. This is orders of magnitude less than that observed using traditional modification. In addition, the insert copy number, insert orientation, the exact sequence of *inserted* DNA, coding and non-coding sequences, and *inserted* gene expression, are fully characterized. All of these factors minimize the risk of the modified tomato becoming a pest. Because the introduced genetic material has been well-characterized, the traits they confer *can* be scrutinized in a way not previously possible to identify any potential "pest" concerns.

Summary

The pest attributes of a plant species are generally removed during the domestication process. Restoration of a crop's pest potential, through traditional or non-traditional genetic modification, would be difficult. The wide range of variability in the tomato, especially as seen from the mutant stocks and traits introgressed from wild relatives, indicates that it is highly unlikely that any new trait introduced into the tomato would cause it to become a pest.

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Paper 6

TRANSGENIC TOMATOES IN CANADA

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Currently all transgenic plant field trials in Canada must be approved prior to their conduct. There are four types of plants that have been identified as having a possible negative impact to the environment. These are:

a) plants with novel pesticidal tolerances
b) plants with novel pesticidal properties
c) plants with novel pest tolerances
d) plants with novel stress tolerances

To date, approximately 300 trials have been approved in Canada since 1988. In 1992, 201 trials were approved at 81 locations. It is important to note that each country tabulates its trial statistics in a different manner so that it is frequently difficult to make accurate comparisons among trial numbers in different countries.

We have approved two tomato trials in Canada. The first application was for the importation and field testing of tomatoes that were engineered to include a gene for the delta endotoxin from *Bacillus thuringiensis*. Although the trial was approved, the company elected to not conduct the trial. The second trial involving a tomato engineered to include novel tolerance to an herbicide was also approved in 1990.

All trials must be conducted in a manner that contains the distribution of the seed and the pollen. Machinery must be cleaned on-site to control seed spread and the disposal of the plant material. The resulting progeny are controlled so that they cannot be sold as seed (including common seed) and cannot enter the human food chain unless a safety assessment has been completed by Health and Welfare Canada. The trial must be reproductively isolated from any plant material with which the transgenics could cross. In the case of tomato, this represents only other cultivated tomato fields. The most common isolation method is the use of spatial isolation; for tomato, the minimum distance requirement is 100m. The land on which the trial is conducted must be kept free of the same or closely related species for a period of years following the trial in order to monitor for dormancy and persistence. In the case of tomato, the post harvest land use restriction period is one year. Trial sites are inspected by Agriculture Canada staff both during and following the trial to ensure that the terms and conditions of the authorization are fulfilled.

In addition to review under the Seeds Acts, trial applications are simultaneously reviewed for compliance with the Plant Protection Act and the Pest Control Products Act. The appropriate provincial agencies are also notified to inform them of the trial and to allow them the opportunity to express any questions or concerns they have with respect to the trial.

Agriculture Canada develops brief environmental screening documents outlining the basis on which approval was granted. Although they are not published, they are available to the public upon request. Agriculture Canada does have provision to maintain certain information respecting the trial as confidential business information. This includes the exact location of the trial, the plasmid map and the exact genes inserted into the transgenic plant.

Agriculture Canada is currently considering the data requirements and decision making criteria that will be used for field trials under unconfined conditions. Prior to commercialization, transgenic agricultural crop varieties will be subject to the provisions of the existing variety registration system. In some cases, the requirement for a food safety assessment will be incorporated into the registration mechanism. The food safety assessment will be conducted by Health and Welfare Canada.

Paper 7

COMMERCIAL TESTING OF TRANSGENIC TOMATOES

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A transgenic tomato variety, whether resistant to an herbicide or devoid of a ripening enzyme, must be tested like any other new variety to assure its performance in all agronomic, grade, transport, factory yield and factory quality characteristics. It must show a net improvement in these areas before it will be accepted by processors and growers.

A typical industrial testing procedure involves 1 to 2 acres at various locations, in grower strip trials the first year, followed by 5 to 10 acre experimental trials the following year. If analysis of these trials shows the variety to be an improvement over a currently used variety of the same maturity and disease resistance, it will be commercially distributed to growers through seed dealers.

However, during the grower trial testing period, it must be acknowledged that control of the seed and pollen becomes limited. In order to conduct the first phase of grower trials, 8-10 pounds of seed are required per company. For tomato hybrids this generally means overseas production. The hybrid seed production can be a very controlled procedure, but it is beyond the boundaries of USA regulations.

Potential for gene transfer

Natural cross pollination of processing tomatoes in commercial fields does occur in California at the very low rate of .0007 to .4080% (Groenewegen, 1990), which is much lower than previous literature (Currence, 1942; Richardson 1956). In the commercial environment, this would not seem to allow for gene escape as any recipient non-transgenic tomato will either be harvested and processed or disked into the soil after harvest. The only inadvertent transfer of transgenic stock that seems plausible would be the case where seed was saved from an adjacent variety in the same field as a transgenic strip trial or test acreage.

Tomatoes that are disked into the soil may well germinate next year as weeds in another crop or as volunteers in a repeat tomato field. Again, the transgenic material will either be suppressed as a weed or harvested as a crop.

Other forms of disposal for transgenic fruit and seed from a commercial trial, are factory pumice, wet waste and mud. These waste products become cattle feed, chicken feed, soil conditioner and soil amendment. Only in the soil amendment category is it likely for seed to germinate, as a contaminant of factory mud. This soil amendment is spread on fallow land and eventually disked in after drying.

Environmental consequences of gene transfer

Future transgenic tomatoes can be expected to improve quality and processing characteristics and possibly disease and herbicide resistance. None of these characteristics are viewed as detrimental to the environment should they occur in tomato plants growing as weeds from soil amendments, volunteer plants from previous fields or load spills while in transit. These sources of non-planted transgenic tomatoes are viewed as more likely to occur than natural cross pollination of the genes into surrounding non-transgenic tomatoes.

Prevention of gene transfer

There is no practical method to prevent the very low frequency of natural cross pollination, and thus gene transfer via pollen. Given the current transgenic characteristics under development, there is no need to prevent such natural low frequency cross pollination. These characteristics do not represent a hazard to either the environment or the processing tomato industry.

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Paper 8

TRANSGENIC TOMATOES: SAFETY AND ENVIRONMENTAL CONCERNS

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Tomatoes as compared to other field crops offer the greatest immediate potential for cultivar improvement through the strategy of genetic engineering. Tomatoes constitute the largest fruit crop and second largest non-grain crop in the United States. Tomatoes have been well characterized and thorough genetic and physical maps are available for breeding purposes. Tomatoes are easily transformed to obtain stable transgenic plants as compared to grain crops. Tomatoes are non-indigenous to the continental U.S. and do not readily outcross to related species. A significant number of physiological processes and genes have been characterized in tomato permitting the rapid improvement of cultivars via genetic modification. Realization of improvements in flavor, color, fiber content, disease and insect resistance, and shelf-life will occur over the next 2 to 5 years. Some of the genes that will be utilized in genetically improved tomatoes will be polygalacturonase and β-mannanase for the manipulation of ripening and soluble solids, *Bacillus thuringiensis* (*B.t.*) toxins for insect resistance, tobacco mosaic virus (TMV) coat protein for resistance to TMV infection, ACC synthase and ethylene oxidase for the synchronization of ripening and improvement of post-harvest storage, peroxidases and phenoloxidases for the modification of phenol metabolism (browning, etc.) and disease resistance, dehydrins for the improvement of salt and drought tolerance, carotenoid biosynthetic enzymes to improve color, and alkaloid biosynthetic enzymes (the expression of which will be suppressed to improve flavor).

Suppression of outcrossing and gene transfer will be less difficult with tomato than with native temperate crops with numerous related weed species such as *Brassica*. Tomato, *Lycopersicon esculentum*, and related non-cultivated species, are native to the Andes region of South America. In cooler climates of the Northern United States the only potential outcrosses will be to other cultivated tomatoes. A border crop of unrelated species of greater than 150 feet should be sufficient to minimize outcrosses. Even though outcrosses from transgenic tomatoes to other cultivated tomatoes or related wild species could be limited through cultivation practices, there remains a remote possibility for escape. For this, there are highly sensitive methods for the detection of outcrosses. These methods include the use of reporter genes in transgenic plants that express enzymes that are easily detectable in trace amounts. These genes are physically linked to the gene of interest, and are not present in higher plants. Examples of the genes include firefly luciferase and E. *coli* β-glucuronidase. Sub-nanogram levels of these enzymes can be detected in large samplings of nearby plants. Another useful method would involve the polymerase chain reaction to amplify transgenes in minute levels. Certainly, current technology would permit the detection of outcrosses, even if these events occur at a low frequency.

Seed from fresh market tomatoes would be difficult to prevent from being sown by the consumer. Also, seed produced as waste from processing may survive sewage treatment and germinate elsewhere down the line. If seed are produced from genetically-engineered tomato plants used for processing, the seed found in the residue must be adequately treated with heat, chemicals, or radiation to prevent germination after disposal. Seed from processing plants can end up in composted sewage used in gardens and landscapes. There have been cases where homeowners have spread composted sewage on their lawns, which soon resulted in a field of tomato plants. Composting methods do not adequately kill tomato seeds. Processing plants would require major renovations to limit viable seed reaching the municipal sewage system. Without such renovations there is an inevitability that viable seed will find its way to soil and germinate. A better solution would involve the use of seedless tomatoes.

Several academic and commercial laboratories are investigating genetic methods of producing hybrid tomato fruit with no seed or non-viable seed. There is also a significant potential for the use of parthenocarpy in the development of transgenic tomato cultivars. Traits such as parthenocarpy and male-sterility have been observed in several tomato cultivars; however, the commercialization of these lines has been limited. Previously, the primary objective in breeding programs has been to improve fruit set in greenhouse-grown tomatoes, since tomatoes only set fruit in a narrow temperature range. The enormous financial incentives with transgenic tomato crops could drive further research into the development of seedless tomatoes. The value of such a crop could also justify the added expense of vegetative propagation, and this method of propagation carries with it the added value of protecting proprietary cultivars. Many genes that control plant hormone production have been cloned, and there have been several reports that demonstrate the ability to alter hormone levels in transgenic plants. This may prove effective in producing sterility during production, yet permit adequate seed production.

In geographical regions with large scale production of transgenic tomatoes there is the potential for increased selection for resistant pests based on introduction of disease and insect resistance genes. It is to be expected that the introduction of a gene such as the *B.t.* toxin into tomato plants will increase the occurrence of insect resistance; however, the frequency of resistance will be dependent on the ecology of the region farmed. Only within the last year has evidence of *B.t.* resistance been revealed, and specific predictions regarding resistance rates appear to be preliminary. The environmental consequences of resistance to *B.t.* will also depend on the regional dependence on microbial *B.t.* formulations and *B.t.*-engineered plants for insect control, and the role of indigenous Bacillus *thuriengensis* in soils for controlling insect populations in the native ecology.

It is my opinion that the commercial success of genetically-engineered crops will require that safeguards be minimized or eliminated. This is a major question about attitudes and perceptions of biotechnology on the whole. The Food and Drug Administration recently agreed to forego labeling indicating genetically-altered, as opposed to non-genetically-altered, foods. This was a necessary step to persuade public acceptance of the products of biotechnology. If growers are required to handle engineered crops substantially different than their current crops i.e., akin to handling toxic waste, this will portray an image to the consumer that this delicious tomato they are about to eat is at the very least special and perhaps dangerous. Also, if growers are besieged with an inordinate number of rules and precautions for engineered crops, this generates a concern about their safety and dramatically increases their costs for production. The long-term success of this technology will require that engineered crops be deemed safe and handled with minimal or no differences at the production level.

In conclusion, the key to public acceptance lies in education and minimizing regulations, and the keys to control involve the production of seedless cultivars and the use of proper cultural practices to minimize outcrossing during production.

Paper 9

TYPES OF FIELD TRIALS AND OUTCROSSING RATES

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Them are two classes of field scale experiments - those for commercial uses (seed production and fruit production) and those for basic research uses (where the desired product might be either fruit, seed, or both).

In addition to transgenes for herbicide resistance and insect and virus resistance, genes modifying fruit ripening characteristics or soluble solids are being tested towards eventual commercial use. The recently developed genetically engineered male sterility system (Mariani *et al.*, 1992) for hybrid seed production may be widely used in tomato. Except for hybrid seed production (which is currently accomplished by hand emasculation and bulk pollinations), fruit production and most basic research applications take advantage of the self-pollinating nature of tomato to produce fruit and seed; therefore, containment of transgenic tomatoes will be easier than that for outcrossing crops.

Because *Agrobacterium* mediated plant transformation relies on identifying transformed cells by virtue of their antibiotic resistance marker, antibiotic resistance genes will of necessity be incorporated into transgenic plants. Use of a site-specific recombination system (such as the cre-lox system) to remove undesired antibiotic resistance genes (Dale and Ow, 1992) could be considered if there is concern about antibiotic resistance in the product. The resulting transgenic plant for commercial use would carry the transgene of interest, but no antibiotic resistance markers.

Many basic research experiments could be more easily carried out in large scale field trials than in greenhouses. The majority of such experiments are unlikely to be performed adjacent to commercial fields, so the risk of spread of transgenes is minimal to zero. Transposon tagging experiments (Osborne *et al.*, 1991) for genes influencing mature plant traits will require field scale experiments. Screening large transgenic populations for second site mutations would most easily be done in the field. For example, seed of antisense polygalacturonase plants (Smith *et al.*, 1988) could be mutagenized and screened for genes that influence expression of the transgene. Transgenic plants harboring genes predicted to influence stress responses could be tested for performance under a variety of environmental conditions.

It is not likely that transgenic tomato pollen will spread to wild species in commercial production fields, inasmuch as wild species of tomatoes are not weeds. It is possible that such spread could occur to the species *L. pimpinellifolium* or *L. cheesmanii* in geneticists or breeders fields, depending on insect pollinator activity. Spread to other wild species from *L. esculentum* is highly unlikely because of breeding barriers, i.e., the wild species must be the male in crosses with *L. esculentum* for the cross to succeed. Spread to other cultivars of *L. esculentum* could happen depending on insect activity, but is not very likely for most tomato cultivars, since they have inserted stigmas and outcrossing is minimal. In any event, a reporter gene could be included to assess spread of the transgene into border rows of non-transgenic tomatoes. It is probable that commercial companies have already performed such tests.

It seems to me that emasculation, deflowering or bagging of inflorescences is not practical for tomato, and is probably not necessary to stop the spread of transgenic pollen. Crop rotation and removal of volunteer plants should be standard practice.

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Paper 10

ASSESSMENT OF RISKS AND TRANSFORMED PLANTS

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This workshop is considering what safeguards are needed for the planned introduction of transformed plants, specifically genetically engineered tomatoes, into field environments. A number of the questions under consideration at this workshop will be specific to the target crop. A case in point is the question of the possibility of unplanned transfer of the gene to wild or weedy species. As will be discussed in this session, the probability of escape is extremely low due to the reproductive features of this crop species, and, in the United States, due to geographic isolation from close relatives.

The answers to some of the questions under consideration should essentially be the same regardless of the target crop. There have been three prior workshops, which focused on crucifers; corn and wheat; and potatoes, respectively; and a larger forum considering all crops held under the auspices of the National Agricultural Biotechnology Council. Therefore this workshop panel can look to the prior workshops for detailed consideration of questions for which the answers are not crop specific. A case in point is the question of what risk is inherent in the features common to plants transformed by the Ti-mediated system. The common features usually consist of T-border regions at the ends of the inserted segment of DNA, and a marker used for selection following transformation, generally an antibiotic resistance gene. The Ti border regions already exist in the general environment, in strains of *A. tumefaciens* that are pathogenic on most of the crops under consideration. It can therefore be argued that the impact of introduction of the same sequences in a transformed plant would be extremely small. The question regarding antibiotic resistance genes is more complex, as discussed in prior workshops. However, at some point a decision must be made. We must decide, as a society, that the potential release of the resistance gene is not a significant risk, devise strategies that disable the resistance gene after transformants have been selected, or else forgo using transformed plants commercially.

Perhaps the most critical decision to be made concerns the basis upon which the risk of a transformed plant is to be assessed: should it be assessed on the basis that it is a transformant, or on the basis of the specific gene introduced? One aspect of this question not specifically addressed in the workshop materials is whether there are certain types of genes, which by their nature, are extremely unlikely to cause any harm even if released. Examples of genes which, in my opinion, are very unlikely to cause harm would be reporter genes constructs expressing ß-glucuronidase (GUS), and plants carrying such constructs. If the decision regarding transformants is made that the critical factor in risk assessment is the target gene inserted, instead of the fact that the plant is transformed, then the current restrictions regarding growing, storing, shipping and general handling of transformed materials are of questionable value in considering the reporter gene constructs. The regulations do not reduce risk if significant risk does not exist. However the regulations do cause loss in terms of time and money spent by researchers and state and federal agencies in implementing these regulations.

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Paper 11

SAFEGUARDS FOR TRANSGENIC TOMATOES

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Summary

As of August, **1992,** over **60 permits** have been issued to conduct field trials in **ten** states with **tomatoes** genetically engineered using one or more of **20** different transgenes. Without exception, all trials have been conducted safely without incident. Given **our** knowledge and experience in field trialing, with genetic engineering and with the behavior of transgenes stably integrated in the **tomato genome,** we *can* now draw conclusions **on** the **safety** of and **necessary** safeguards for transgenic tomatoes trials:

1. Because of outcrossing barriers, flower morphology, and **a** high incidence of self-pollination, the impact of any specific transgene will be localized within the trial itself. The transgene must be thoroughly evaluated to determine whether it **poses** any risk *to* altering the nature of **tomato** prior to uncontained release. In assessing the possibility of transgenic **tomatoes** or **a** sexually compatible weed pest becoming a greater weed problem, it is important to consider that weedy properties usually represent complicated, multigenic **traits** and generally do not result from single gene **traits** (Keeler and Turner, **1991).** Regardless, any effect of a transgene will be limited to *Lycopersicon esculentum* and not affect other *Lycopersicon* or *Solanum* species.

2. Horizontal gene flow is not an issue for **tomato;** such an event **has** not **been** demonstrated for any transgenic crop and **no** known mechanism **has** been proposed.

3. The use of the *Agrobacterium*-mediated transformation system and the regulatory regions from *Agrobacterium* and cauliflower mosaic virus do not **pose** risks in transgenic **tomatoes because** all infectious and hazardous regions of these microorganisms were eliminated prior to transformation.

4. For almost **all** situations, gene transfer from transgenic **tomato** trials *can* be prevented. Straightforward, simple cultural practices *can* be used to prevent gene transfer.

Tomato Taxonomy and Genetics Support the Conclusion that Outcrossing to Other Species Will Not Occur

Tomato *(Lycopersicon esculentum* Mill.) is a member of the *Solanaceae* family which includes potato and **tobacco.** Cultivated **tomato** is one of nine *Lycopersicon* species (Rick, **1978),** all of which have the **same** number of chromosomes **(2n** $= 2x =$ 24) and chromosome morphology (Rick, **1976).**

Esquinas-Alcazar (1981) describes the **natural** range of *Lycopersicon:*

The **natural** distribution of the genus *Lycopersicon* extends from northern Chile to southern Colombia and from the Pacific coast (including the Galapagos islands) to the lower eastern foothills of the Andes. Many species overlap but **no** evidence of natural introgression **has** been found, with the exception of *L. pimpinellifolium* and *L. esculentum.* All the species have well-defined **ranges** of distribution, except *L. esculentum* var. *cerasiforme* (cherry **tomato)** which is the only wild and weedy *Lycopersicon* found outside the **area** of distribution of the genus. It is also present in the Old World where it might have **escaped** cultivation.

No wild *Lycopersicon* **has been** found outside Latin America, except for the very uniform *Lycopersicon esculentum* var. *cerasiforme.*

The pre- and post-fertilization barriers that prevent cross-pollination **between** *Lycopersicon* species **are** well documented (Rick, **1979;** Taylor, **1986).** Tomato *can* only **be** crossed by hand-pollination to wild *Lycopersicon* species. The genus **has** been divided into **two** subgenera, one which crosses easily with commercial **tomato** (*esculentum* subgenus), and the other which does not (*peruvianum* subgenus). The *esculentum* subgenus consists of *L. esculentum, L. cheesmanii, L. chmielewskii, L. hirsutum, L. parviflorum, L. pimpinellifolium,* and *Solanum pennelli.* The *peruvianum* subgenus consists of *L. chilense* and *L. peruvianum.* Wide hybridization between members of the two subgenera usually leads to early embryo breakdown and nonviable seed. Sexual hybridization between the two subgenera can only be accomplished using embryo culture. The closest genetic relatives of *Lycopersicon* are in the genus *Solanum. L. wculentum* can also be crossed with *S. lycopersicoides* using controlled pollination techniques, although the hybrids are usually sterile (Stevens and Rick, 1986). Attempts to cross *L. esculentum* with *S. rickii* and *S. ochranthum* failed (Rick, 1979). Recently, a controlled cross between *L. wculentum* and *S. rickii* was successful using a sesquidiploid bridging hybrid (De Verna *et* al., 1990), which may provide a means to move genes from *S. rickii* to commercial cultivars. No other member of the genus, including *S. nigrum*, a common weed in tomato fields, has yielded any viable hybrids with tomato (Taylor, 1986).

Resistance to 14 pests has been bred into commercial cultivars from wild *Lycopersicon* species using controlled crossing techniques. For example, the gene for *Fusarium* resistance came from *L. pimpinellifolium* and the gene for root knot nematode resistance came from *L. peruvianum* (Rick, 1983). Other examples of fungal resistance bred into cultivated tomato are: resistance to early blight, anthracnose and *Verticillium* wilt from *L. esculentum* var. *cerasiforme*; and resistance to botrytis mold from *L. hirsutum* (Esquinas-Alcazar, 1981). Resistance to curly top virus came from *L. chilense* (Esquinas-Alcazar, 1981). Similarly, improvements were made in high soluble solids in fruit using crosses with *L. chmielewskii* (Rick, 1983). Genes that prevent easy fruit abscission and retention of pedicels came from *L. chmielewskii* (Esquinas-Alcazar, 1981).

Although these reports indicate that crosses between *L. esculentum* with all *Lycopersicon* species within the genus can be achieved, natural interspecific crossing is at least confined within the tomato's natural range in South America and only within the *wculentum* subgenus. There is strong evidence, however, that even in the natural range, interspecific crossing does not occur. Esquinas-Alcazar (1981) states that many species overlap but no evidence of natural introgression has been found, with the exception of *L. pimpinellifolium* and *L. esculentum.*

Cultivated tomato is self-fertile. Although it outcrosses to a considerable extent in its native region and certain other subtropical areas, elsewhere it is almost completely self-pollinating (Rick, 1976). This autogamy is a result of transition in cultivated tomato from exserted to inserted stigmas within the anther cone (Rick, 1979). Over the past 50 years, the change in style-length has been dramatic, which further improved self-pollination and consequent fruit set and practically eliminated outcrossing (Rick, 1976). Taylor (1986) reports, all representatives of *L. esculentum* are selfcompatible and exclusively inbreeding.

These observations support the conclusions that gene transfer from transgenic tomatoes to related species will not occur and that gene transfer to other tomato plants will be rare.

Tomato Does Not Have Weediness Characteristics

Assessment of weediness potential should be done at two levels. The first is a determination of whether transgenic tomato is itself a weed pest or is sexually compatible with weedy relatives. For tomato, neither is true. However, this conclusion could be different if the nature of the transgene were such that certain characteristics of tomato changed. Then a second level of assessment would be needed to examine specific properties of the transgenic tomato, particularly those that are generally attributed to weeds, such as seed dormancy, long soil persistence of seeds, germination under diverse environmental conditions, rapid vegetative growth, a short life cycle, high seed output, high seed dispersion, long-distance dispersal of seeds, etc. (Baker, 1974).

Three aspects of weediness (Keeler, 1989) are of concern for transgenic tomato:

1. Comparison of transformed crops with exotic species. Is the experience with introduction of exotic plants into new

environments (thereby creating a weed problem) a valid analogy for introduction of transgenic tomatoes?

2. Potential for transformed, domesticated tomato to revert to a weedy state. Are them examples in which tomato has become weedy, such as due to plant breeding or movement outside its center of origin?

3. Potential for hybridization between tomato and wild relatives creating or dancing weediness *(see* also previous section).

Likelihood of Tomato Becoming a Weed: Comparison of Transformed Crops with Exotic species

The analogy between introduction of an exotic species into a new environment and introduction of a transgenic crop is tenuous (Fincham and Ravetz, 1991). Introduced exotic plants that have become pests bring with them many traits that enhance weediness and, very importantly, leave behind control organisms (predators) and competitors. Transgenic plants are altered in only a few, specific characteristics that relate to crop production and food quality characteristics (National Research Council 1989). Unlike exotic plant introductions, transgenic tomato will generally not be released into exotic environments, but will be planted within the existing production range of cultivated tomato. Tomato has been introduced and grown throughout the world without it becoming a weed pest.

For the most part, introductions of exotic species have been environmentally harmless and economically beneficial; most North American crop plants are in fact exotic. In rare cases, such as kudzu, introductions have resulted in environmentally undesirable consequences. In most such cases, careful review of the organism's biology would have predicted the unfavorable consequences (Williams, 1980) and the problem of weediness could have been avoided. In like manner, careful consideration of the biology of a transgenic tomato will alleviate any concern that it might respond like an exotic species that becomes established as a weed pest.

Since tomato is an exotic species in most countries and has not become a weed pest, the model of exotics becoming pests upon introduction into a new environment is inappropriate for tomato.

Potential for Tomato to Become a Weed Pest

Tomato is not listed as a weed in the major weed references (Crockett, 1977; Holm *et al.*, 1977; Muenscher, 1980), nor is it present on the lists of noxious weed species distributed by the State of California and the Federal Government. Although *L. esculentum* var. *cerasiforme* has become established in the wild in south Florida and southernmost Texas, it is not considered a weed pest. Furthermore, there is almost no probability of transgenic tomato naturally introgressing into var. *cerasiforme*. C. Rick's view of gene transfer is that the risk of such introgression is nil or almost nil (pers. comm.).

By using a variety of plant breeding techniques, tomato varieties have been continually selected for improved resistance or tolerance to external factors that inhibit their inherent productivity. Tomato varieties have been selected for insect and disease resistance, better tolerance to environmental constraints to growth (such as heat, cold and drought), and ability to prevail in competition with weeds through quick germination and extremely rapid growth in the seedling stage. In theory, such improved cultivars are better adapted to persist in the presence of disease, insects, and environmental constraints. However, tomato breeders have a long history of incorporating these types of traits into crops without evidence of enhanced weediness (United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS); 1991).

Similarly, it can be expected that tomatoes modified by molecular and cellular methods should present no different risks in regards to weediness potential. Since molecular methods are highly specific in terms of which genes are being added, users of these methods will be more certain about the traits they introduce into plants (USDA, APHIS; 1991) and the weediness potential may actually be less than using traditional breeding methods. Nevertheless, it is important to consider the effect of new, introduced genes on the potential for tomato to become a weedy pest.

For new genes to be retained in a tomato population, the genes must have at least one of three characteristics (Hauptli *et al.*, 1985): (a) they must confer improved fitness to the first and resultant generations of the species; (b) they must have no negative effect on fitness; and/or (c) the genes must be tightly linked to other genes conferring improved fitness.

According to Keeler (1989), tomato does not have the following traits that characterize weedy species: (a) internally controlled, discontinuous germination; (b) long-lived seed; (c) very high seed output; (d) perennial life cycle and ability to be vegetatively propagated, (e) difficulty in uprooting; (f) good competitive ability; (g) polyploidy; and (h) reported as a weed.

In general, tomato lacks many of the traits characteristic of weed pests and has not been considered a weed pest in the United States. The USDA has concluded in environmental assessments of transgenic field trial applications that tomato does not display significant potential to develop into a weed itself (USDA, APHIS; 1991).

Potential for Hybridization Between Tomato and Wild Relatives Creating or Enhancing Weediness

Of significance is the lack of weed pest relatives of tomato. *Solanum nigrum* is the only major weed pest related to tomato (Holm *et al.*, 1977; Lange *et al.*, 1986). Other members of the nightshade family which are weeds in tomato fields are: *S. sarrachoides, Physalis heterophylla, P. lanceifolia, P. xocarpa, P. acutifolia, Nicotiana bigelovii, Datura stramonium, D. meteloides*, and *D. ferox* (University of California, 1985). Other weedy *Solanaceae* are: *Hyoscyamus niger, Lycium ferocissimum, P. virginiana* var. *sonorae, P. viscosa, S. cardiophyllum. S. carolinense, S. dimidiatum, S. dulcamara, S. elaeagnifolium, S. lanceolatum, S. marginatum*, and *S. torvum* (Lorenzi and Jeffery, 1987).

L. esculentum is sexually incompatible with all of these weedy relatives. Because tomato has no weed pest relatives, there is no possibility of a cross between tomato and wild species that would enhance weediness.

Plant-to-Microorganism Gene Flow Does Not Occur

Another potential mode of gene escape from tomato is the possibility that a gene would not remain immobile (stably integrated in the tomato genome), but would migrate from its chromosomal location in the tomato cultivar, and take up residence in some other organism, such as a microorganism. Such movement is termed horizontal transfer. The possibility of horizontal transfer is of concern such as when addressing antibiotic resistance genes, because of the potential to expand the population of antibiotic resistant pathogens. Arguments have been made concerning this potential risk, but no data have been published to support such a concern.

Horizontal gene transfer from tomatoes does not represent a risk, because no mechanism for transfer of genes from plants to microorganisms is known and no cases of such transfer have been reported. Carlson and Chelm (1986) argued for an eukaryotic (plant) origin of glutamine synthetase II in bacteria, albeit over an evolutionary time period. They suggested that this was evidence that horizontal gene flow from plants to microorganisms had occurred at one point in evolution. However, their paper was directly refuted by Shatters and Kahn (1989) who concluded that the GS [glutamine synthase] proteins are highly conserved and the divergence of these proteins is proportional to the phylogenetic divergence of the organisms from which the sequences were determined. No transfer of genes across large taxonomic gaps is needed to explain the presence of GSII in these bacteria. Other evidence that horizontal gene flow occurs from plants to microorganisms involves transient changes (non-heritable) such as transencapsidation of chloroplast DNA (Rochon and Siegel, 1984) or possibly endocytosis (Bryngelsson *et al.*, 1988), neither of which has been shown to result in actual transfer of genes from plants to microorganisms. No mechanism by which plant DNA could be incorporated into the genomes of the microorganisms has been proposed. In addition, Zambryski *et al.* (1982) provided evidence that once inserted DNA is integrated into the plant host genome, it cannot be remobilized even if acted on again by *vir* genes. To date, such horizontal gene flow remains speculative with no actual examples.

Donor Genes from Plant Pest Organisms Do Not Pose a Risk

The Ti plasmid from *Agrobacterium*, used to produce most transgenic tomatoes, is disarmed so that the plasmid no longer can redirect plant cells into biosynthesis of phytohormones leading to gall formation. This is done by constructing a plasmid that does not contain the phytohormone (*onc*) genes. The Ti plasmid contains the T-DNA that is stably integrated into the plant nuclear genome. Because none of the T-DNA genes are involved in transfer and integration (Zambryski, 1988), this integrated material does not contain the necessary *A. tumefaciens* genes, such as the *vir* genes needed for transfer and infection (Fincham and Ravetz, 1991).

Functions of a native (fully armed) Ti plasmid that are not transferred to transgenic tomato are the *vir* and *onc* genes, the nopaline or octopine catabolism genes (*nos* and *oct*), the ability for conjugal transfer of the Ti plasmid between bacteria (*tra* functions), and origin of replication and other replication functions (Hohn and Schell, 1987; Koukolíková-Nicola *et* al., 1987).

Following the use of *Agrobacterium* for tomato transformation, the *Agrobacterium* are killed with carbenicillin so no subsequent infection or transformation can occur (Fillatti *et al.*, 1987). The transgenic tomatoes are then grown to flowering, and seed is collected for future generations and field production. Because of these procedures, the original plant transformation vector (Ti plasmid) does not remain associated with the plants, and any further transfer of genes from such plasmids to humans, animals or the environment can not occur.

The 35S promoter region (CaMV35S) is derived from cauliflower mosaic virus (Gardner *et* al., 1981). Cauliflower mosaic virus is a double-stranded DNA caulimovirus with a host range restricted primarily to cruciferous plants. Genome size is about 8 kb. CaMV35S has a very high constitutive strength as compared to other plant promoters, allowing it to be widely used as a promoter for high expression of genes (Gronenborn and Matzeit, 1989).

CaMV35S has not been shown to be a plant pest risk in plants. Palukaitis (1991) concludes that, while some of these plants [containing CaMV35S promoter] may have shown either unusual or abnormal *responses*, it has in every *case* been possible to delimit these host abnormalities to the expression of the gene and not to the presence of a promoter of viral origin. There is no evidence that the sequences of the CaMV promoters are in themselves inducers of pathogenicity. Thus, the major CaMV gene products, rather than the well-characterized regulatory signals on CaMV DNA, are involved in the induction of pathogenicity in plants.

Safeguards for Conducting Transgenic Tomato Field Trials

Probably 90% of the concern about transgenic tomatoes relates to potential effect on tomato as food and whether any nutritional or potential toxins have been altered, thereby changing its food safety. Such concerns can be dealt with through analytical techniques (Redenbaugh *et al.*, 1992); however, discussions on food safety are beyond the intent of this paper, which is focused on the concerns relating to effect on the environment.

From the information above on taxonomy/outcrossing and experience with transgenic tomatoes, it is clear that the principal concern in conducting transgenic tomato trials is the inserted transgene and whether, as part of the tomato, it poses a risk to the environment should an inadvertent release occur. The focus of any risk assessment should then be on the transgene and its effect on tomato. The tomato genus *Lycopersicon* is itself not a weed pest risk. Outcrossing with relatives will not occur because of natural barriers, leaving only the issues of pollen movement from the transgenic trial to other tomato plants and seed survival at the trial site.

Based on these discussions and the 60 field trials of transgenic tomatoes in the U.S., several basic components can be identified which will minimize or eliminate any possibility of gene escape from contained field trials. These recommendations for conducting transgenic field trials are as follow:

1. Maintain a 30-foot isolation zone from other tomato fields to prevent

outcrossing. The isolation zone can be barren, planted with a non-tomato crop, or planted with a tomato border that will be destroyed and treated in the same fashion as the transgenic trial.

2. During the growing season, visually inspect equipment used in the trial and remove any plant material, leaving the debris at the trial site.

3. Conduct harvesting operations separately from adjacent tomato fields. All plant debris, particularly reproductive material, should be maintained on site, except for fruit and seed saved for further evaluation and planting.

4. After harvest, disk and water the field. Monitor the field and control volunteers, as necessary, for 6 months.

5. Conduct seed processing operations separately from other processing activity.

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Paper 12

NATURAL CROSS-POLLINATIONIN TOMATOES

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The tomato is generally classified as a self-pollinated crop. The structure of the normal tomato flower promotes autogamy: anthers are arrayed in a tube, into which pollen is released, drifts distally, and is directed toward the stigma. As anticipated, exserted stigmas (long-styled pistils) are more prone to outcrossing whilst those that are depressed within the tube (short-styled pistils) are subject to nil or extremely little outcrossing. Position of the stigma is influenced by both genotype and environment, although the impact of the former is fortunately stronger and readily manipulable (Rick and Dempsey, 1969). The extent of outcrossing *can* also be strongly modified by such other genetic alterations of floral structure as male sterility, absence of stamens, failure of attachment of stamens, and other defects (Rick and Robinson, 1951).

Another major factor influencing rates of outcrossing is the activity of various bees (usually not including honey bees), which are the principal pollen vectors. In the U.S. bumble bees and solitary bees are the main agents, but in the tropics and subtropics, particularly in the native Andean area, carpenter bees, stingless bees and other groups of bees also participate in this activity (Rick, 1950). Under field and greenhouse conditions airborne pollen is of little consequence, as repeatedly evidenced by lack of fruit set on emasculated or male-sterile flowers in situations that exclude bees but permit air circulation. Attractivity of flowers is a prime factor affecting visitation by pollinators. Thus, in our fields, solitary and bumble bees seldom visit flowers of cultivated tomatoes but are often attracted to the larger, better displayed flowers (usually with well-exserted stigmas) of certain related wild species.

Rates of natural outcrossing can be estimated experimentally by testing the proportion of hybrid progeny or, more directly, by observing the extent of fruit and seed set on genetically male-sterile plants. In either method plants of the essential genotypes are interplanted with normal, fertile plants in plot designs appropriate for measuring outcrossing. Hybrid progeny in the former method are detected by use of readily detected monogenic markers usually detected in seeds (via allozymes) or seedlings (ex: anthocyanin deficiencies), and the outcrossing rates estimated from the frequency of dominant phenotypes in the resultant progeny. Marker genes must not modify flower structure or otherwise affect insect visitation. In the use of male-sterile mutants, the proportion of flowers setting fruit and numbers of seeds per fruit expressed in terms of isogenic fertile plant yields provide the desired comparisons. As expected, rates based on the latter method tend to be higher than with the former because male-sterile flowers do not produce functional pollen to compete with transmitted pollen, yet both methods are useful for comparing rates in different seasons, genotypes, habitats, etc.

In general, both methods generally reveal very low rates of outcrossing in the North Temperate Zone and higher rates in the tropics and subtropics, particularly in the native Andean region of *Lycopersicon* spp. The range of rates measured in the U.S. varies from 0-5%, lower values prevailing. Our tests in the Andes indicated at least 10-fold higher rates via the *ms* method (Rick, 1950); about 30-fold higher rates via genetic detection of hybrid progeny (Rick, 1958). Higher rates in the native region would be anticipated because the pollen vectors essential for reproduction of the sympatric, self-incompatible, strictly allogamous, wild tomato species are vastly more active there. Our tests within California also revealed areas of higher crossing rates in plots close to nesting sites of solitary bees (Capay, Oceanside) but generally not close to areas of tomato fruit or seed production (Rick, 1949).

Experiments on other factors have also been reported. Genotype of either parent may influence outcrossing rates (Soost and Rick, 1957). Dramatic reductions are observed in genotypes with depressed stigmas which, incidentally, are better fruit setters by virtue of more effective self-pollination (Rick and Dempsey, 1969). Thus, we have rarely observed outcrossing to large open-pollinated progenies of our standard line of the short-styled cv. VF36. Tests on isolation by distance have generally revealed a marked reduction in rates with increasing distance between parents (Currence and Jenkins, 1942). In our rather limited tests (Rick, 1947). rates were far higher for close (15 cm) plantings than for plants spaced 1-2 m apart.

Please note that the research reviewed above deals mostly with pistillate aspects of outcrossing. It is these aspects that are of greatest interest to the plant breeder and seedsman because they affect: (1) requirements for isolation to prevent contamination by cross-pollination; and (2) exploitation of natural cross-pollination for hybrid seed production. The extent of exit of pollen from tomato plantings – the main theme of this workshop – has received little attention. Nevertheless, the findings and conclusions reached in the aforementioned studies are valid as they apply to cultivated tomatoes. They do not deal directly with the extent of crossing from cultivated tomatoes to wild or feral taxa as pistillate parents. It is important to point out, however, that much experimentation has been performed on controlled reciprocal hybridization between cultivated tomato and related wild species (Rick, 1979) and that in the majority of combinations, a strong unilateral barrier obstructs crosses in which the latter are pistillate parents. These crosses usually fail in prefertilization stages, primarily as a result of blocked pollen tube growth. In combinations with more distantly related taxa (species of *Solanum* and other genera), such barriers are absolute. For these reasons alone, the risks of gene escape via pollen from tomato plantings are minimal.

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Paper 13

TRANSGENIC TOMATO FIELD TESTING CONSIDERATIONS

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Potential for gene transfer

Tomato, *Lycopersicon esculentum* Mill., is known as a self-pollinating vegetable requiring only enough isolation distance to prevent mechanical mixing during planting or harvest for seed production (Lorenz and Maynard, 1980). Pollination is primarily by wind but insect transfer sometimes occurs under field conditions (Rick, 1958; Quiros and Marcias, 1978). Cultivated tomatoes have perfect flowers with stigmas within the anther cone, which limits outcrossing. This is in contrast to most related *Lycopersicon* species which have exserted stigmas more conducive to cross-pollination. The amount of cross-pollination in tomato has been the subject of several papers and results have varied from 1 to 43% (Quiros and Marcias, 1978) although generally less than 4%. The amount of cross-pollination is contingent on several factors. One is field location which is important due to both weather conditions, which can can cause style elongation (stigma exertion), and local insect populations, which might vector pollen from one plant to another. The other important factor is the neighboring tomato genotypes, which may vary in resistance to style elongation. Several papers report on environmental conditions which induce style elongation (see Scott and George, 1980). These include high temperature, high nitrogen, low light intensity, short days, low humidity, and low soil moisture. All 'these conditions could result in low carbohydrate accumulation in the plant which could trigger the style elongation. Tomato breeders always select for yield, and in so doing, select for stable, non-exserted stigma positions since yield problems often result with stigma exsertion (Scott and George, 1980). In my 11 years in Florida, I do not recall ever seeing stigma exsertion on varieties in commercial fields. Without stigma exsertion, the possibility of transgenic pollen landing on such stigmas by wind is probably zero or extremely rare, and by insects (bees) nearly as rare.

Quiros and Marcias (1978) found that three genera of bees pollinated tomatoes in Celaya, Mexico. One of these, *Bombus sonorus* (bumble bee), is the only bee or insect I have ever seen working *Lycopersicon* in Florida and they much prefer wild species to cultivated tomatoes.

Thus, pollen transfer in commercial fields would be extremely rare but could be greater in areas where neighboring tomatoes might have exserted stigmas. Even then, if the fruit with seed fertilized by escaped pollen was eaten, gene escape would not be likely. Rick (1958) found a fair amount of crossing in Peru where growing of transgenic tomato varieties would be more subject to outcrossing. Several *Lycopersicon* species would still not be affected because tomato will only cross with them in the opposite direction (i.e., with the transgenic tomato as the female parent).

Rick (1979) has summarized crossing relationships within the *Lycopersicon* genus. Interspecific crosses between tomato and other *Lycopersicon* species are often difficult or impossible. No crossing with the closest related genus, *Solanum*, has been reported (Rick, 1979). Thus, escape of transformed genes to weeds would be impossible in my estimation since there are no *Lycopersicon* weeds.

Seed from fruit left in the field, with either hand or mechanical harvest, would seem to be the more likely method of gene escape. These would result in volunteers in the field which should be controllable with herbicides or by disking. A severe flood could carry fruit out of the production area where seed might germinate.

Environmental Consequences

If transformed genes escaped, the potential effects would depend on the genes involved. Current transgenic tomato development involves genes that affect herbicide resistance, insect resistance (*Bt*), disease resistance (viral and fungal), fruit composition (often ripening), and male-sterility. These are listed from most to least "dangerous", but I don't believe the danger is serious for any. Herbicide resistant tomatoes could pose more of a weed problem, but tomatoes presently are not a weed problem even when herbicides are not used. Any factor that would increase survivability could increase possible weed hazards including disease or insect resistance. However, any escaped plants with increased fitness due to these factors would be of most concern as volunteers that could harbor disease or insect pests, rather than as weeds competing with a crop. However, it is doubtful that such plants would cause a major upset of an ecological system. Adding disease resistances by conventional breeding has not had any drastic environmental effects in over 50 years. Conventional breeding for insect resistance has not been nearly as successful as breeding for disease resistance, but I know of no problems of such genes escaping from unprotected breeding plots and causing environmental problems. It is hard to imagine how the fruit composition transformants would cause more damage than the *rin* gene, which has been tested in fields for over 20 years without a problem. Male-sterile transformants would have lower fitness in the environment than a normal tomato.

A future area for genetic engineering might be related to stress adaptation. These modifications might result in tomatoes that might be more of a weed problem, but such a prediction could be tested. Any widely adopted "silver bullet" gene system could lead to a genetic vulnerability problem, but this could also be the case with non-transformed genes. An area of possible concern is that a genetic construct could be transferred to other microbes resulting in unforeseen environmental consequences. Others can speak to this better than I can.

Safeguards

In a Mexican study, 100 meters was considered a safe isolation distance to avoid pollen contamination (Quiros and Marcias, 1978). This was in agreement with earlier studies. The distance would probably not have to be so great in most U.S. production areas due to lesser wild bee activity. Stress conditions could result in more outcrossing if neighboring varieties had exserted styles. This could be studied. Other than fallow ground, borders of wild *Lycopersicon* species that do not cross with tomato could limit pollen flow by bees since they would be more attractive to them. However, such species might bring the bees into the area and have an opposite, undesired effect. Tall windbreaks would also limit pollen escape. None of the other suggestions listed in the handout are practical with tomato. Mesh cages could limit bee activity and reduce pollen spread by wind for small scale testing. However, this wouldn't be very practical in obtaining field information.

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Paper 14

SOME CONSIDERATIONS ON THE POSSIBILITY OF HORIZONTAL GENE TRANSFER FROM GENETICALLY ENGINEERED TOMATOES TO BACTERIA

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As genetically engineered tomatoes are grown more widely in open field environments, the possibility of gene transfer from transgenic tomatoes to other organisms is an area of increasing interest. Studies have demonstrated that tomato can cross with related *Lycopersicon* species (Rick, 1975), so the possibility of gene transfer to weedy *Lycopersicon* taxa has been a subject of considerable concern. Indeed, this is reflected by the focus of many papers in the current workshop on planned introductions of transgenic tomatoes.

Much less is known about the possibility of horizontal gene transfer from tomato (or any other higher plant) to microorganisms. Even in well-characterized systems like *Agrobacterium* and *Rhizobium* there has been little or no consideration of the possibility. Nonetheless, gene transfer from tomatoes or other higher plants into microorganisms is plausible and worthy of study. This type of horizontal gene transfer could potentially have an impact on the use of transgenes in agriculture, as well as the overall nature of plant-microbe ecology and evolution.

How Likely is Gene Transfer From Tomatoes to Bacteria?

While gene transfer from higher plants to bacteria seems very unlikely to occur, there have been no studies to confirm this point. Conditions that might be favorable for gene transfer from tomatoes to bacteria frequently occur. For example, tomato plants are often attacked by bacterial pathogens, including *Pseudomonas solanacearum* (bacterial wilt) and *Clavibacter michiganensis* subsp. *michigunensis* (bacterial canker), as well as the tumor-forming, *Agrobacterium tumefaciens* (crown gall). Many of these pathogens reside exclusively within the plant body. Here, dying cells would be expected to release significant amounts of partially degraded plant genetic material. It is well-known that *Agrobacterium* can transfer DNA from its *Ti* plasmid into the genome of plant cells (reviewed in Zambryski, 1988) — now we need to know whether DNA can go in the opposite direction.

Given an opportune environment for gene transfer into bacteria, how likely is it to take place? Bacteria would first have to be competent to take up foreign DNA. The evidence strongly suggests that many bacterial taxa can become naturally competent, including genera with species pathogenic on tomato (Stewart, 1989). Moreover, the conditions required for natural competence are often not so different from the milieu likely to be found inside a tomato plant (Carlson *et al.*, 1983).

Assuming successful introduction of plant DNA into a bacterium, the foreign DNA would have to be incorporated into genomic or plasmid DNA to be stably maintained. This would require that the foreign DNA survive the restriction/modification defense mechanisms of the bacterium. However, because plant genomic DNA tends to be methylated, restriction/modification may not be an effective barrier and foreign DNA might well survive.

Still, there would normally not be any regions of homology between tomato DNA and that of bacteria, so recombination into the bacterial genome would typically occur at quite low frequency (though other integration mechanisms that require only short regions of homology might still be possible). However, if DNA from transgenic tomato plants were taken up into bacteria, homologous DNA associated with genetically engineered sequences would probably exist, particularly *Agrobacterium*-derived DNA sequences. This would increase the possibility of stable incorporation significantly. Thus, if natural transfer of plant DNA into bacteria occurs at all, it might be more likely to occur with transgenic plants.

Experiments to Test Horizontal Gene Transfer

Given the possibility of gene transfer from plants to bacteria and the nearly total lack of information in this area, it is useful to consider experiments to test the hypothesis. A good place to start would be to see if bacteria that naturally attack tomato can take up foreign DNA in culture (particularly the type of DNA sequences that might be used in genetic engineering) without special attempts to make the bacteria competent. Of course, some selectable marker would have to be included in the test DNA molecule. It would be important to use a marker that is not bacterial in origin to rule out the possibility of gene transfer from microbial contaminants. The selectable marker could then be flanked by sequences typically used in plant genetic engineering (like sequences from the T-DNA region of the *Ti* plasmid) and which would be expected to promote recombination into bacterial or plasmid genomes. These experiments would be safe and simple, and potentially quite informative.

Assuming that exogenous DNA can be taken up in culture by naturally competent tomato bacterial pathogens, the next step would be to look at transgenic tomato plants. One might transform into tomato the same type of DNA sequences used in the culture experiment, sequences with a selectable, but non-bacterial marker, joined to suitable bacterial DNA sequences to promote recombination. Plants would then be inoculated with the phytopathogenic bacteria, which would later be isolated out of the plant, cultured on a selective media and probed for diagnostic sequences. This should indicate whether genetically engineered DNA has moved from the plant into bacteria.

Environmental Consequences of Horizontal Gene Transfer

Assuming that rare gene transfer events from transgenic tomatoes into bacteria occur, the new transgenic bacteria would have to compete effectively with native populations to be of ecological concern. Moreover, to expand in the ecosystem, the transgene would have to confer increased fitness to the recipient individual. Many of the genes now being considered for genetic engineering into tomato, such as delayed ripening, would hardly be expected to confer enhanced fitness on microorganisms. It is more difficult to predict the consequences of transferring genes for herbicide tolerance or resistance to tomato pathogens and insect pests.

Nevertheless, any transgene incorporated into a bacterial genome might very likely be transferred into other microorganisms in the environment. This could occur through any of several horizontal gene transfer systems known to exist among bacteria, which would be even more rapid if the transgene was incorporated into a plasmid (Amabile-Cuevas and Chicurel, 1992). Potentially, tomato transgenes transferred into bacteria might even resurface in non-*Lycopersicon* plant taxa. Clearly, the likelihood of these scenarios are extremely low, but the essential point is just how little we know about the probabilities of these events in nature.

Safeguards to Horizontal Gene Transfer

As little as we know about the potential for gene transfer from tomatoes to bacteria (let alone the consequences), it is difficult to establish rational safeguards. A few measures are obvious and might go a long way to limit this type of gene transfer, if it does indeed exist. The first would be to minimize the use of bacterial sequences used to transform tomato plants. This would limit the opportunities for recombination of tomato transgenes into bacterial genomes. Another important measure would be to minimize the opportunities for bacteria to take up the DNA in the first place — in other words, keep tomatoes healthy and free of the bacteria most likely to take up the transgenic DNA, perhaps through the use of varieties resistant to bacterial diseases. Finally, genes transformed into tomato could be designed with introns and other distinctly eukaryotic signals, minimizing the chances they would be expressed even if successfully transformed into bacteria.

Conclusions

The possibility of gene transfer from higher plants to bacteria is an area that needs to be studied further. It is clear that this type of DNA transfer is conceivable — what we do not know is whether it actually occurs in nature, and if so, how frequently. This type of information is even more essential as scientists begin to release transgenic tomatoes (and other transgenic plants) into the environment. Even as the possibility of gene transfer to bacteria is analyzed, so should the possibility of gene transfer into fungal and viral plant pathogens, as well as naturally occurring endosymbiotic organisms. The consequences of gene transfer into microbes may turn out to be minor, but we will not know for sure until we know more about the process itself.

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