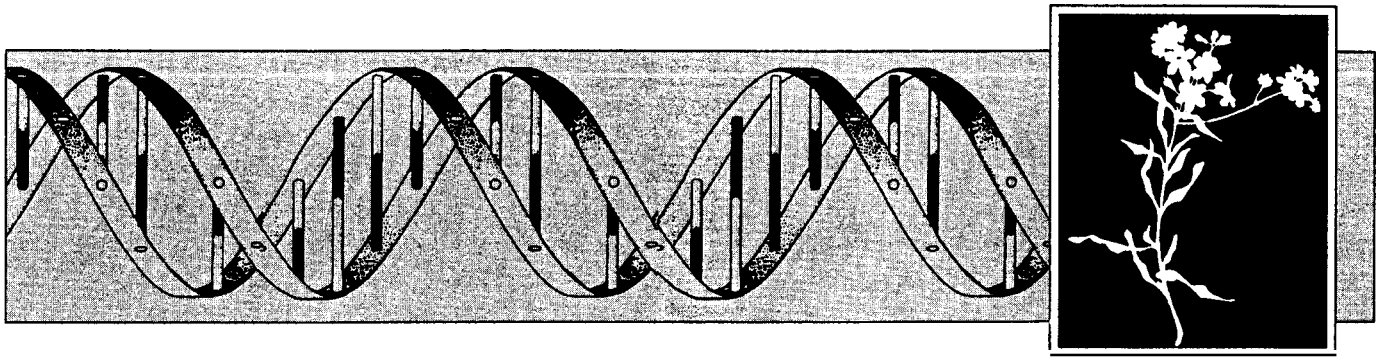
Section III.



Sample Application for Release Into the Environment

Questions on the Preparation of Application for a Field Test

Comments about issues that ***may*** need to be addressed in this section are enclosed in boxes.

1. Is the person whose name appears in box 1 on APHIS Form 2000 (see form on the next page) also the person who has signed on line **14?**

2. Is the application typed on ***8*** 1/2" by 11" paper?

To save space in this guide, copy is printed on both sides of the paper. However, permit applications must be typed on only one side.

3. Is the text of the application organized into nine sections according to points stated on line 13 a–i on APHIS Form 2000?

4. Are the pages numbered by their section number followed by their page number (e.g., 13c–12)?

5. Does the application contain any confidential business information (CBI)?

If the answer is no, do the first pages of both copies have the phrase "NO CBI"?

If the answer is yes, is there a CBI copy and a CBI-deleted copy?

6. Are the CBI and CBI-deleted copies prepared according to the following points?

a. On each page containing CBI material, is the CBI material designated by a bracket and the term "CBI" in right margins next to where the material is located, and is the phrase "CBI COPY located on the upper right corner of the page?

b. Each page with CBI-deletions should be marked "CBI-deleted" in the upper right corner of the page. In the right margin, mark the place where the CBI material has been deleted with a bracket and "CBI-deleted."

c. The CBI-deleted copy should be a facsimile of the CBI copy, except for spaces occurring in the text where CBI has been deleted. Additional material (transitions, paraphrasing, generic substitutions, etc.) should not be included in the CBI-deleted copy. **If several pages are CBI-deleted, a single page stating each deleted page may be substituted for several blank pages.**

d. Published literature usually cannot be claimed confidential and thus must appear in both copies.

e. If any information in an application is claimed as CBI, the applicant must support each of these claims by including a written justification.

Examples of pages containing CBI and CBI-deleted material are shown on pp. III-28 and III-29.

7. Reprints should not be submitted with the application unless they provide specific information required for addressing statements 13 a–i (e.g., a sequence of a gene or genetic map). Any *preprints* of publications cited in the permit application should be included if possible and may be claimed CBI.

Sample Application: Release Into the Environment

1 NAME AND ADDRESS OF APPLICANT
Dr. Edward Johnson
Paige-Sullivan Biotechnologies, Ltd.
6505 Belcrest Road, Hyattsville, MD 20782
Area Code ( )

2 PERMIT REQUESTED (*X* one)
- [ ] Limited Interstate Movement
- [ ] Limited Importation
- [X] Release into the Environment
- [ ] Courtesy Permit

3 THIS REQUEST IS (*X* one)
- [X] New
- [ ] Renewal
- [ ] Supplemental

4 TELEPHONE NUMBER
(301) 436- 7612

5 MEANS OF MOVEMENT
- [ ] Mail
- [X] Baggage or Handcarried
- [ ] Common Carrier

By whom Company employees

6. GIVE THE FOLLOWING (if applicable) (if more space is needed, attach additional sheet)

	Scientific Name	Common Name	Trade Name	Other Designation
a Donor Organism	Cucumber mosaic virus (CMV)			
b Recipient Organism	Lycopersicon esulentum cv. Packard Clipper (tomato)			
c Vector or Vector Agent	Agrobacterium tumefaciens and Ti plasmid			
d Regulated Organism or Product	tomato expressing CMV coat protein			
e If product, list names of constituents				

7 QUANTITY OF REGULATED ARTICLE TO BE INTRODUCED AND PROPOSED SCHEDULE AND NUMBER OF INTRODUCTIONS
enclosed

8 DATE (or inclusive dates of period) OF IMPORTATION, INTERSTATE MOVEMENT, OR RELEASE
May 15, 199X

9 COUNTRY OR POINT OF ORIGIN OF THE REGULATED ARTICLE
USA

10 PORT OF ARRIVAL, DESTINATION OF MOVEMENT, OR SPECIFIC LOCATION OF RELEASE
Hyattsville, MD

12. APPLICANTS FOR A COURTESY PERMIT. STATE WHY YOU BELIEVE THE ORGANISM OR PRODUCT DOES NOT COME WITHIN THE DEFINITION OF A REGULATED ARTICLE

13. SEE REVERSE SIDE

I hereby certify that the information in this application and all attachments is complete and accurate to the best of my knowledge and belief.

False Statement: Falsification of any item on this application may result in a fine of not more than $10,000 or imprisonment for not more than 5 years or both (18 U S C 1001)

14 SIGNATURE OF RESPONSIBLE PERSON	15. PRINTED NAME AND TITLE	16. DATE
Ed Johnson	Ed Johnson, Regulatory Affairs Officer	10/29/9X

Srale Notification Sent	State Review Received	Permit Issued ☐ Yes ☐ No

Date of Determination	Permit No.	No of Permit Labels Issued	Supplemental Conditions Enclosed ☐ Yes ☐ No
Signature of BBEP Official		Date	Expiration Date

APHIS FORM 2000 (JUL 89) *Replaces PPQ Form 1001 which may be used.* *(continued on reverse)*

	ENCLOSURES	ENCLOSED ("X")	IF PREVIOUSLY SUBMITTED, LIST DATE & PERMIT NO.
a	Names. addresses, and telephone numbers of the persons who developed and/or supplied the regulaled article	X	
b	A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the non modified parental organism (e g , morphological or Structural characteristics, physiological activities and processes, number of copies of inserted genetic material and the physical slate of this material inside the recipient oryanism (integrated or extrachromosomal). products and secretions, growth characteristics)	X	
	A detailed description of the molecular biology of the system I e g , donor. recipient-vector) which is or will be used to produce the regulated article	X	
d	Country and locality where rhe donor organism. recipient organism. arid vector or vector agent were collected. developed and produced	X	
e	A detailed description of the purpose for the introduction of the regulated article including a detailed description of the proposed experimental and/or production design	X	
f	A detailed description of the processes. procedures. and saleguards which have been used or will be used in the country of origin and in the United States to prevent contamination, release, and dissemination in Ihe production of the donor organism. recipient organism. vector or vector agent, constituent of each regulated article which is a product. and. regulated article	X	
g	A detailed description of the intended destinalion (including final arid all intermediate destinations) uses and/or distribution of the regulated article (e g greenhouses laboratory or growth chamber location. field trial location pilot project location production propagation. and manufacture location, proposed sale and distribution location)	X	
h	A detailed descriplion of the proposed procedures processes and sateyuards which will be used to prevent escape and dissemination of the regulated article at each of the intended destinations	X	
	A detailed description of the proposed method of final disposition of the regulated article	x	

Public reporting burden lor this collection of information is estimated to average 5 hours per response, including the time for reviewing instructions. searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions lor reducing this burden. to Department of Agriculture, Clearance Officer, OIRM, Room 404-W, Washington, D C 20250. and lo the Office of Information and Regulatory Affairs, Office of Management and Budget, Washington. D C 20503

APHIS FORM 2000 (Reverse)

13a. Names of persons who developed genetically engineered organism

The transformed plants were developed by:

Dr. Ed **Johnson**
Paige-Sullivan Biotechnologies, Ltd.
6505 Belcrest Road
Hyattsville, MD 27082
(301) 436-7612

Dr. M. C. Halasa
Halasa Plant Products
P.O. Box 1948
Minot, North Dakota
(401) 436-7777

If resumes, curriculum vitae, or other personal information are submitted in the application, they may be claimed as confidential business information (CBI).

13b. Description of Regulated Article

The genetically engineered tomato plants have been developed to express the coat protein gene of cucumber mosaic virus (CMV). The rationale for this experiment is given below.

PROBLEM: Viral diseases are one of the major limiting factors in tomato production. CMV is one of the most frequently detected viruses in tomatoes (Benner et al. 1985) and is an especially severe problem in China, Indonesia, and Japan. To date, no effective gene for resistance has been identified in Lycopersicon spp. by plant breeders. Therefore, we have attempted a novel approach to obtain a form of viral resistance by incorporating a gene into the tomato genome that will synthesize the CMV coat protein.

BACKGROUND: Cross protection is the mechanism whereby infection of a plant by one strain of a virus protects the plant from the effects of subsequent inoculation with another strain of the same virus (Nton 1982). Cross protection was first demonstrated by McKinney (1929). Tobacco plants infected with a green mosaic virus (a TMV strain) did not develop further symptoms when inoculated with a yellow mosaic virus strain. Thung (1931) confirmed these experiments and was unable to isolate the second virus from doubly inoculated plants. This suggested that the second virus had not multiplied. Salaman (1933) found that tobacco plants inoculated with a mild strain of potato virus X were "immune" from subsequent inoculation with severe strains of the virus, even if the challenge was performed 5 days after inoculation. The infected plants were not immune to infection with unrelated viruses: tobacco mosaic virus or potato virus Y. Some viruses do not appear to induce cross protection at all (e.g., curly top virus of sugar beets). Most experiments on cross protection have been carried out using mechanical transmission, but cross protection has also been demonstrated with viruses that are transmitted in a persistent manner by insect vectors (Harrison 1958). Several theories have been put forth that explain the cross protection phenomenon. (1) The first strain uses some essential metabolite required by the second strain. (2) The virus-infected plants produce "protective substances" that inhibit replication of the challenge virus. Although inhibitory substances are detected in virus-infected plant extracts, the evidence does not support that these compounds are involved in the cross protection phenomenon. (3) Kavanau (1949) suggested that "aggregates" of virus in cells previously infected with a virus have some specific "adsorptive properties." Others (De Zoeton and Nton 1975, Sherwood and Nton 1982, Matthews 1982) have suggested that the viral coat protein could be the "adsorptive" molecule. They have proposed that when the challenge virus is uncoated, it is rapidly reencapsidated by viral coat protein synthesized by mild strain. This theory is consistent with observation that *only* closely related viruses show cross protection phenomenon. The first definitive evidence for a putative role of viral coat protein in the cross protection-like phenomenon was use of the transgenic plants expressing viral coat proteins.

GENETICAUY ENGINEERED CROSS PROTECTION In 1986, Abel et al. produced a transgenic tobacco plant which expressed the TMV coat protein gene. Upon challenge with whole virus, plants expressing the gene showed a delay in symptom development and, in some cases, plants failed to develop symptoms for the duration of the experiment. The authors called this phenomenon "genetically engineered cross protection." Challenge inoculation with viral RNA rather than virus largely overcame the protective effect. This leads to the conclusion that the presence of the coat protein on the virus particle in challenge inoculum was necessary for maximum protection and that encapsidation of naked challenge RNA by coat protein was not involved in the protection phenomenon. Recently, Nelson et al. (1988) reported that if partially stripped TMV virions were used as the challenge inoculum, the protective effect was largely overcome. (Partially stripped virions have coat protein subunits removed, exposing approximately 150 nucleotides of the 5' end of viral RNA.)

This suggests that coat protein interferes with disassembly of the incoming challenge virions. It is not known if classical and genetically engineered cross protection are based on similar mechanisms.

Other workers have engineered plants that synthesize the coat protein of alfalfa mosaic virus (Loesch-Fries et al. 1987, Turner et al. 1987, Van Dun et al. 1987), and tobacco rattle virus (Van Dun et al. 1987). The results from these experiments further confirmed that plants expressing coat protein genes interfere with viral multiplication.

Recently, the effectiveness of "genetically engineered cross protection" in a field situation was demonstrated (Nelson et al. 1988). In field-grown plants, less than 5 percent of the coat protein expressing plants inoculated with TMV exhibited visual symptoms by fruit harvest as compared to 99 percent of the challenged control plants. Fruit yield reduction was approximately 30 percent due to virus infection in the nonengineered controls as compared to coat protein expressing plants.

Traditional cross protection phenomenon between CMV strains has been demonstrated by Dodds and coworkers (1982, 1985). Cuozzo et al. (1988) have produced transgenic tobacco plants expressing CMV coat protein that are protected from challenge inoculation by a severe strain. Paige-Sullivan Biotechnologies has cloned and sequenced CMV coat protein and produced transgenic plants that produce high levels of CMV capsid protein (White 1988). The viral coat protein has been introduced into tomato by the leaf disc transformation technique (McCormick et al. 1986).

13c. Description of Donor, Recipient, and Vector

Two approaches were utilized to transform plants: A. tumefaciens and the Ti plasmid or electroporation.

DESCRIPTION OF THE VECTOR SYSTEM: The vector system used to transfer the coat protein gene of cucumber mosaic virus (CMV) to tomato plants is based on the Ti plasmid from Agrobacterium tumefaciens. The vector system is "disarmed" or nonpathogenic because all the genes involved in phenotypic expression of the disease characteristics have been deleted. We have utilized a "two-component" system of transferring genes into tomato leaf sections. This system was chosen for its use in cloning genes of interest into plasmids and for greater transformation frequencies with certain plant species and/or cultivars and was developed by Deblaere et al. 0985).

In using this system, the scientific literature supports the view that *only* the T-region is transferred and integrated into the plant genome (Fraley et al. 1986, Cooper and Meredith 1989). The sequence that is integrated includes the genes contained between certain short, well-characterized segments of the Ti plasmid that are essential for incorporation into the plant genome. *Also,* border sequences (25 base pairs required for transfer) are lost during the process of insertion of T-DNA into plant genome. This means that the inserted DNA is no longer a functional T-DNA capable of being transferred by the same mechanism that originally inserted the T-DNA into plant genome (Zambryski et al. 1982). Thus, all evidence available since the delineation of T-DNA in 1978, plus the accumulated information concerning the epidemiology of crown gall disease, indicates that T-DNA transfer into plant cells by Agrobacterium is irreversible.

CONSTRUCTION OF ACCEPTOR PLASMID: The purpose of this procedure is to obtain a Ti plasmid lacking all of the T-region DNA but retaining the vir region. *An* octopine plasmid pTiB6S3 containing two adjacent T-regions (T_L and T_R) was modified to contain a kanamycin resistance marker. The intermediate vector pGV746, a pBR322 derivative, contains two Ti plasmid sequences that are located respectively to the left and outside the T_L DNA segments and to the right and outside of the T, DNA sequences. A double recombination between pGV746 and pGV2217 results in pGV2260 (fig. 1). In pGV2260 the entire T_L and T_R regions are deleted and substituted by sequences derived from pBR322.

CONSTRUCTION OF THE VECTOR PLASMID: The initial plasmid pGV700 is a pBR322 derivative containing 1 kb HindIII/BglII part of HindIII-18 fragment of pTiAch 5 and the 6.5 Kb BglII/HindIII part of HindIII fragment of pTiAChS (see fig. 2). This plasmid contains all T-region sequences except genes 5,7,2, and 1. A 7.5 kb HindIII fragment from pGV700 was recloned into pGV600, giving rise to pGV742. pGV600 is a pBR322 derivative lacking *any* BamHI sites. The remaining T_L DNA sequences (but not T_L 25 bp border sequences) in pGV742 were removed by deleting internal BamHI fragment giving rise to pGV744. The T_R DNA sequences (but not the RB 25 bp sequences) were removed by deleting the internal EcoRI fragment, giving rise to pGV749. To obtain a plasmid containing *only* border sequences, the 1.87 kb HindII/NruI fragment from pGV749 was cloned into pGV710, previously digested with EcoRI/HindIII. pGV710 is a pBR322 derivative containing Sm^R, Su^R, Cm^R, and Tc^R markers. The sticky ends obtained after digestion were flush-ended by treatment with Klenow DNA polymerase prior to HindIII treatment. The resulting plasmid pGV815 was isolated as a Sm^R, Cb^R, Cm^S, Tc^S clone. The EcoRI and HindIII sites of this plasmid were eliminated by filling in the sticky ends and self ligation of the vector. The chimeric kanamycin was produced by inserting a 298 bp BclI/BamHI fragment from pGV230 (which contains a NOS promoter) into BclI site of pKC7 to produce pKC7::NOS. Plasmid pCK7 is a pBR322 derivative containing 1.8 kb

HindII/BamHI fragment of Tn5 which contains NPT II (Rao and Rogers 1979). The NOS 3' polyadenylation and termination signal sequences were isolated and fused to NPT II (NEO) gene as previously described (White 1988). The chimeric NPT II gene was isolated as a BclII/BamHI fragment and cloned into BglII site of pGV825 to produce pGC831.

DESCRIPTION OF RECIPIENT: The recipient organism, L. esculentum cv. Packard Clipper, is a common commercial cultivar and is a fresh market tomato. It is not widely grown in Maryland because of its susceptibility to CMV. Additional information on the biology of this tomato can be found in section 13h.

DESCRIPTION OF DONOR: CMV has been linked to plant disease in all temperate regions of the world. The virus has an extremely wide host range that includes cereals, forages, woody and herbaceous ornamentals, vegetables, and fruit crops. The RNA of CMV consists of four components of different size (approximate M_r: 1.01, 0.89, 0.68, and 0.33 x 10^6). The three largest RNA's, which are distributed among three separate virion particles, carry all the information needed for successful infection. The genetic information for viral coat protein is carried on RNA 3 and on a coencapsidated subgenomic messenger RNA 4 (Kaper 1984). The viral coat protein assembles around the viral RNA to form the stable virion, which proteets the nucleic acid from physical, chemical, or biochemical degradation. The coat proteins from different CMV strains usually have different amino acid sequences that reflect differences in nucleotide sequences of RNA 4 (Kaper 1984).

CMV strain PV 29 (also called strain 1) (American Type Culture Collection Catalogue, 14th Ed., 1988) was propagated as previously described (Lot et al. 1972) and dsRNA isolated as previously described (Diaz-Ruiz and Kaper 1978). Full-length ds cDNA copies of PV 29 CMV RNA 4 were prepared using synthetic oligonucleotide to prime RNA synthesis simultaneously from the 3' ends of both plus and minus strands of denatured ds RNA 4. The primer hybridizing to the plus strand contained an added ClaI recognition site at its 5' end to facilitate forced cloning into the plasmid vector. Additional details of this construct have been published (White 1988), and a reprint appears in appendix 1.

The promoter used in this study was derived from strawberry vein banding virus (SVBV), a caulimovirus (Shepherd 1979). The replication strategy of SVBV is thought to be analogous to the well-characterized cauliflower mosaic virus (CaMV). Upon infection of a cell with SVBV, two major RNA transcripts, designated 40S and 22S (based on their sedimentation coefficients), are produced during the replication of SVBV. These transcripts are analogous to the 35S and 19S CaMV RNAs (Hull and Covey 1983). The 40S SVBV promoter sequences have been characterized (further details in appendix 1). The 405 promoter was isolated as the HindIII/ClaI fragment extending from +9 to -343 with respect to the transcription start site mapped for the 40S RNA. The transcription termination and polyadenylation signal sequences were derived from the nopaline synthase (NOS) gene (Barker et al. 1983).

The 40S promoter and NOS termination signal sequences were blunt-end ligated to the cloned CMV coat protein gene and inserted into pGV831 at the unique BamH1 site. A map of the resulting plasmid pJLW180 is shown in figure 3. pJLW180 was introduced into the acceptor Ti plasmid pGV2260 by a single homologous recombination, using Sm^R gene of pJLW180 as a selectable marker for cointegration (see fig. 4 for map of cointegrate). The mobilization of pJLW180 from E. coli to Agrobacterium C58C1Rif^R (pGV2260) was performed according to Van Haute et al. (1983). The structure of the T-region was confirmed by Southern blot hybridization.

AGROBACTERIUM-MEDIATED TRANSFORMATION Subterminal leaflets from 6-week-old, greenhouse-grown plants were used for transformation as described by McCormick et al. (1986). Regenerated plantlets (R_1) were challenge inoculated with CMV-CQ (10 µg/ml) and placed in a greenhouse. Symptom development was monitored until fruit were harvested (plants were allowed to self-pollinate). A small portion of the seeds, collected from the fruits of three plants showing the mildest viral symptoms, were germinated on kanamycin-selective media. Results of segregation of antibiotic sensitivity suggest that one line pJLW180-110 (R_2) contains a single CMV coat protein loci (table 1). Seedlings from seed lot pJLW180-120 will be used for the field test.

DIRECT TRANSFORMATION (ELECTROPORATION): Tomato protoplasts were isolated from fully expanded mature leaves as described by O'Connell and Hanson (1987). Protoplasts were electrically permeabilized in the presence of 50 µg/ml pJLW180 as described previously (Fromm et al. 1986) except that the electrical pulse was delivered 122- or 245-µF capacitors charged to 200V (Fromm et al. 1986). The electroporated protoplasts were cultured as previously described; callus and subsequent plantlet formation was performed as previously described by O'Connell and Hanson (1987). A total of 113 plantlets were regenerated; 43 were tested for CMV coat protein synthesis by protein dot blot analysis. Of these, 31 were positive (i.e., contained >1 ng of coat protein per µg of protein) for coat protein production (table 2). Protein analysis was performed as described by Nelson et al. (1987).

Table 1. Genetic analysis of progeny of self-pollination of three transformed lines with respect to kanamycin sensitivity

	Kanamycin insensitive	Kanamycin sensitive	Ratio tested	Chi-square*
pJLW180-110	78	22	3:1	0.5 (0.25<P<0.5)
pJLW180-120	140	67	2:1	0.1 (0.75<P<0.9)
pJLW180-160	344	19	15:1	0.6 (0.25<P<0.5)

*Hypotheses were rejected at the 5-percent risk level (P<0.05).

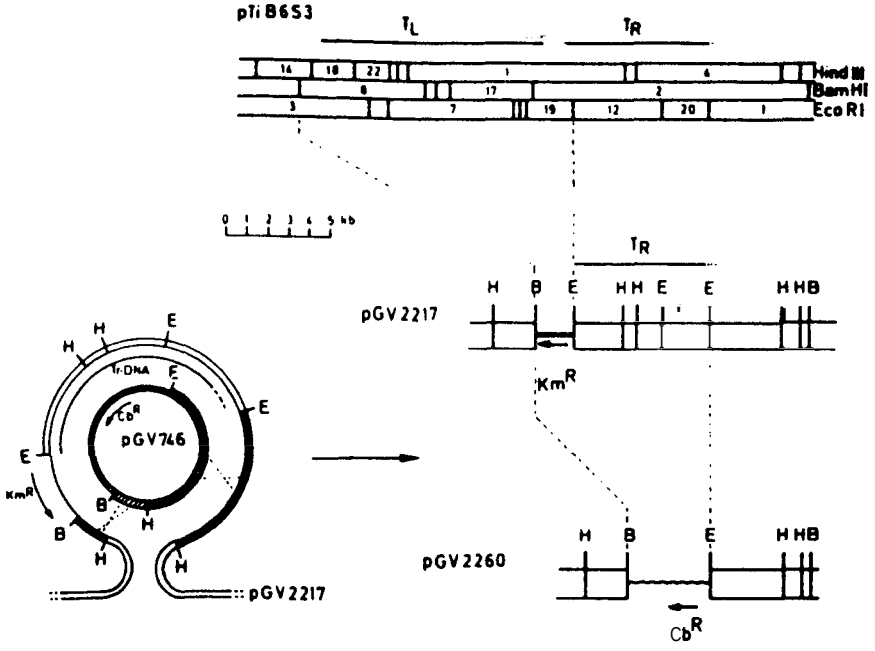
2:1 — Integration of the T-DNA (containing Km^R gene) induces a mutation that is lethal when homozygous: the expected segregation ratio is 2:1.
3:1 — The kanamycin marker segregates as one Mendelian locus, and homozygotes are viable.
15:1 — The kanamycin resistance marker segregates as two independent Mendelian loci.

Table 8. Detection of CMV coat protein in 43 direct transformants of tomato 13c-4

Direct Transformants Designation	Amount of CMV Coat Protein[1]			
	<1 ng	1–5 ng	5–10 ng	>10 ng
12a,12c,12d,12f	+			
12g,12k,12n,12p	+			
12b,12e,12s,122		+		
12y,12w,12r,12q		+		
12j,12s,12t,12u			+	
12m				+
13m,13o,13q,13t	+			
13u,13v,13w,13x		+		
13y,13z,13a,13b		+		
13c,13f,13h,13t		+		
13n,13p,13k,13q			+	
13r,13s				+

[1]After extraction of protein from leaf tissue, 20 μg of protein was subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis (Nelson et al. 1987). The amount of CMV coat protein expressed was based on intensity of band compared to that of known concentrations of viral coat protein.

Figure 1. Construction of pGV2260 (the disarmed Ti plasmid).



Restriction map of the T-region of pTiB6S3 and pGV2217. In pGV2217 [6], the TL-region is substituted by a Km^R marker. The intermediate vector pGV746 was constructed as follows : the 2.3-kb HindIII/BamHI fragment from pTiAch5 fragment HindIII-14 ([///]) was cloned into pBR322, digested with HindIII and BamHI. This fragment is directly adjacent to the $left_S$ of the TL-region. The resulting plasmid, pGV713, was selected as a $Cb^R Tc^S$ clone. The pTi-region adjacent to the right of the TR-region was cloned as a 4.2-kb EcoRI/HindIII fragment, derived from pTiAch5 fragment HindIII-4 ([·.·.]) into pGV713 digested with EcoRI/HindIII. The resulting intermediate $vector_R$ is pGV746. Recombinants between pGV746 and pGV2217 $were_R$ isolated as Cb^R transconjugants after mobilizing pGV746 into $C58C1Rif^R$ (pGV2217) using the technique described [25]. The double cross-over events between $pGV746_R$ and pGV2217, indicated by crossed $lines_R$, were obtained by screening the Cb^R transconjugants for the $loss_R$ of the Km^S marker present on pGV2217. The physical structure of one Rif^R, Cb^R and Km^S transconjugant, pGV2260, was verified by Southern hybridization and is depicted in the figure.

[Figure 1 is reproduced from its original source, Deblaere et al., Nuc. Acids Res. (1985) 13:4777-4788, by permission of Oxford University Press.]

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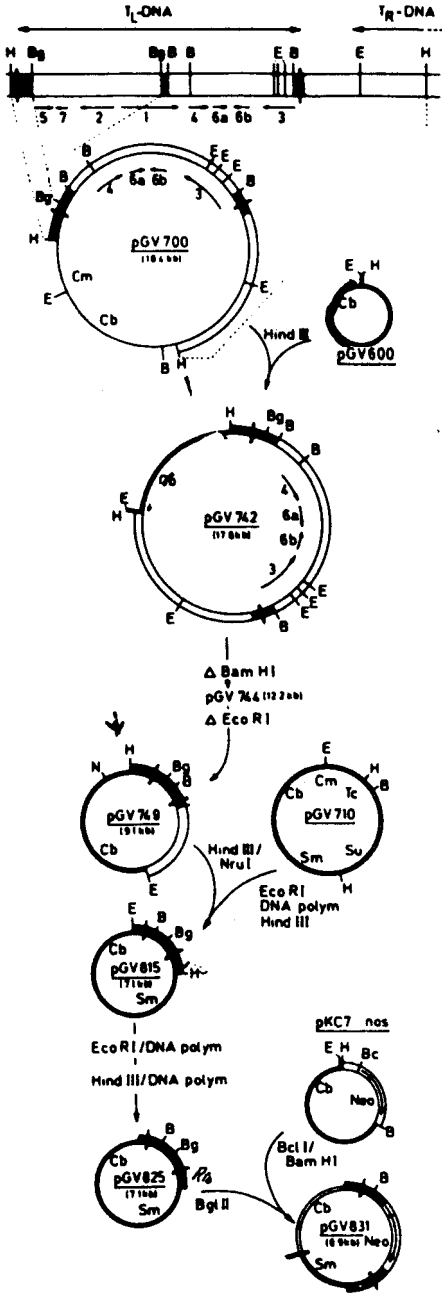
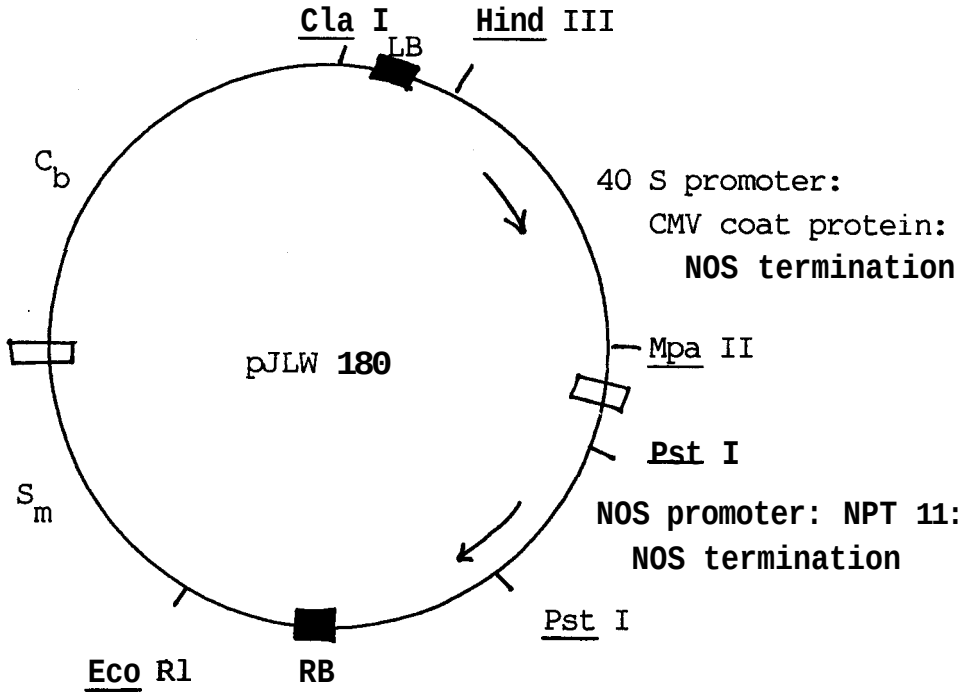
Figure 2. Construction of pGV831.



The T-region of pTiB6S3 is presented on top-of the figure. The dark fragments are those which are maintained in pGV831. The 7.5-kb HindIII fragment from pGV700 (Table 1) was recloned into pGV600, a pBR322 derivative lacking the BamHI site. The remaining TL-DNA genes in pGV742 were removed by deleting the internal BamHI fragments (pGV744). The left part of the TR-DNA was removed by deleting the internal EcoRI fragments (pGV749). pGV710 is a pBR325 derivative that contains an additional $Sm^R Su^R$ marker. To obtain pGV710 the 2.43-kb HindIII/PstI fragment from pBR325, containing the Cm^R gene, was cloned in a HindIII/PstI-digested cosnid pHC79 and the 1.62-kb BglII "cos" fragment of the resulting plasmid was substituted by a 3.45-kb BamHI fragment from the P-type plasmid R702 that encodes resistance to Sm/Sp and Su [31]. In order to obtain a fragment containing only the TL-border sequences, the 1.87-kb HindIII/ NruI fragment from pGV749 was cloned into pGV710 digested with EcoRI, and HindIII. The sticky endsobtained after EcoRI digest were flush-ended by treatment with Klenow DNA polymerase before HindIII digestion. pGV815 was isolated as a Sm^R, Cb^R, Cm^S and Tc^S clone. In pGV825 the EcoRI and HindIII sites were eliminated by filling-in the sticky ends and self ligation of the vector. A 298-bp BclI/ BamHI fragment from pLGV2381 [14] comprising the nopaline synthase promoter and cloned into the BclI site of pKC7 produced pKC7::nos. The nos promoter directs transcription of the neo gene in plant cells [14] This chimeric Km^R gene was isolated as a BclI/BamHI fragment and cloned into the BglII site of pGV825 to produce pGV831. Abbreviations : B, BamHI; Bc, BclI, Bg, BglII, E, EcoRI; H, HindIII; N, NruI; Cb, carbenicillin; Cm, chloramphenicol; Sm, streptomycin; Sp, spectinomycin; Su, sulfathiazol; Tc, tetracycline.

[Figure 2 is reproduced from its original source, Deblaere et al., Nuc. Acids Res. (1985) 13: 4777-4788, by permission of Oxford University Press.]

Figure 3. Map of pJLW180.



S_m - streptomycin resistance marker
C_b - carbenicillin resistance marker
LB, RB - left and right border sequences

The border regions are derived from an octopine type plasmid (Thomashow et al. 1980); the LB (1050 bp) and RB (550 bp). The chimeric CMV coat protein contains the **NOS** termination and polyadenylation **signal** sequences (nucleotides 19,995 to 20,543 (Barker et **al.** 1983)) and the chimeric NPT 11 gene contains the **NOS** promoter (BamHl fragment of pLGV2381 (Herrera-Estrella et al. 1983)) and the **NOS** polyadenylation signal sequences (White 1988). NPT 11 was isolated from Tn5 (Deblaere et al. 1985).

Legible, freehand drawings are acceptable.

Figure 4. Map of cointegrate: pJLW180::pGV2260.

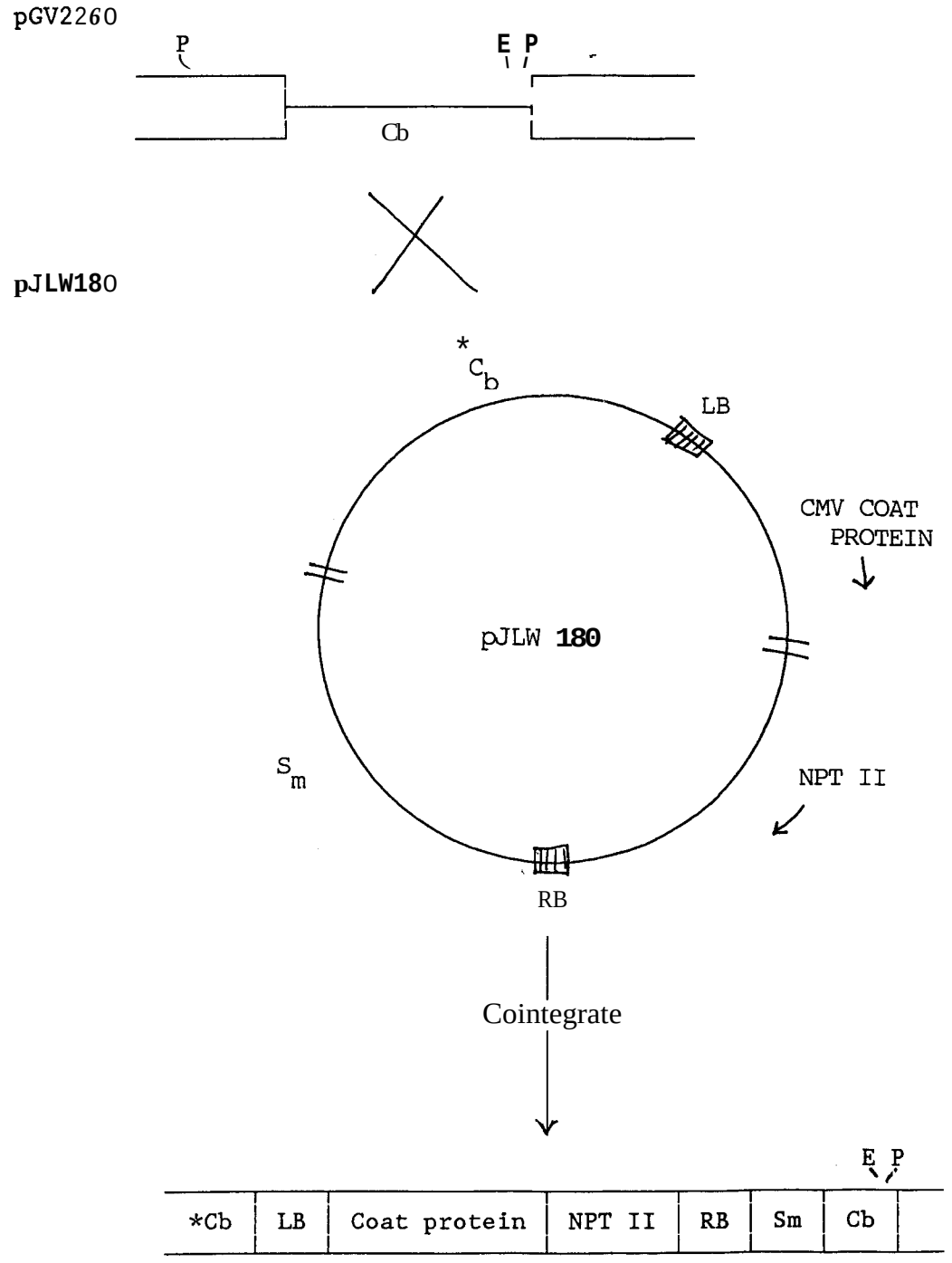
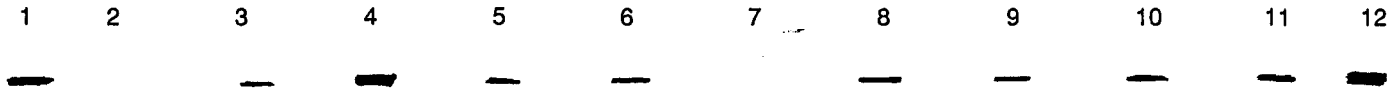


Figure 5.



Autoradiograph showing the DNA analysis of plants transformed with CMV coat protein. Southern blot analysis of HindIII-digested DNA from nine directly transformed plants (lanes 1 to 9) and vector transformed plant (pGV831; lane 10) and probed with ^{32}P-labeled ssRNA transcripts of the cDNA to CMV coat protein. Lanes 11 and 12 contain one or five copies, respectively, of cloned CMV coat protein per genome equivalent.

(1) Is there a detailed description of the derivation (whether or not they are derived from plant pests) of all new sequences that appear in the regulated article, including promoters, polyadenylation and termination signal sequences, the engineered gene(s), marker or antibiotic resistance gene(s), and other noncoding sequences?

(2) Which of the newly acquired genes are expressed in the regulated article?

(3) Is the origin of the vector/vector agent described?

(4) Is there a description of how the vector/vector agent transfers the gene(s) to the recipient?

(5) If the vector/vector agent has been derived from a plant pest, are there any genes remaining that are or have been implicated in pathogenicity of this organism?

(6) Can the mechanism by which the engineered gene(s) were introduced be reversed and mobilize the gene(s) out of the engineered organism to other organisms?

(7) If they can be mobilized out, explain the mechanism and include data, if available, on the frequency and species of organisms that could be potential recipients.

13d. Source of Regulated Article

The transformed tomato plants were developed at Paige-Sullivan Biotechnologies, Ltd., Hyattsville, Maryland. The tomato seeds of cultivar Packard Clipper were obtained from Packard Caribbean Seed Company, Riverside, California. The plasmid used to transfer the CMV coat protein gene to the tomato plants was constructed at Halasa Biotechnologies, Minot, North Dakota. The *A. tumefaciens* strain used was obtained from Dr. Leonard C. Jackson, Dept. of Plant Pathology, University of California, Davis, under USDA/APHIS permit number 88-111-33.

13e. Field Plot Design.

PURPOSE: The objective of this field trial is to test the level of tolerance of plants expressing CMV coat protein against challenge inoculation by severe strain of CMV.

FIELD SITE LOCATION: The company-owned site, in Hyattsville, Prince George's County, Maryland, is surrounded by agricultural land. The crops in adjacent fields at the expected planting date are soybeans and corn. The closest nonexperimental tomato plants are assumed to be at the nearest residential home, approximately 3/4 mile away.

FIELD TRIAL SUPERVISOR: Jack Baker, Field Operations Manager, (301) 436-7612.

EXPECTED PLANTING DATE: May 15.

FIELD DESIGN. Split-plot design with nine treatments as main plots and two replications.

GENOTYPES:

(1) Nontransgenic control Packard Clipper

(2) R_2 progeny of transgenic line pGV831

(3) R_2 progeny of transgenic line pJLW180-120

(4) Direct transformants (R_1) of transgenic line pJLW180-160

TREATMENTS:

(1) Noninoculated - Packard Clipper

(2) Noninoculated - transgenic pGV831

(3) Noninoculated - transgenic pJLW180-120

(4) Challenge inoculated with CMV-CQ - nontransgenic control

(5) Challenge inoculated with CMV-CQ - transgenic pGV831

(6) Challenge inoculated with CMV-CQ - transgenic pJLW180-120

(7) Challenge inoculated with CMV-CQ - transgenic pJLW180-160

Applicants must name the county or counties where the field test(s) will be performed.

Seeds will be germinated in the greenhouse. Plants will be "hardened off" in a cold frame for up to 1 week prior to transplanting in the field after the permit for this application is issued. All challenge inoculations, which will be done mechanically, will take place 10 days after transplanting. CMV strain CQ used in this study is endemic to the Eastern United States.

If a pathogen to be used in the field test was received under a PPQ 526 movement permit, a copy of the permit should be submitted along with this application. If a plant pest is being moved interstate for the field test (e.g., CMV strain *CQ* for challenge inoculation in this sample application), a permit may be required (PPQ Form 526). This form can be obtained from Biological Assessment and Technical Support staff, USDA-APHIS-PPQ, Room 625,6505 Belcrest Road, Hyattsville, MD 20782.

PLOT DESIGN Each plot contains 20-ft rows with 20 plants spaced 1 ft apart and 5 ft spacing between rows. The main plot will consist of two experimental rows and two border rows on the outside (total four rows). There will be a 20-ft unplanted area between all main plots to prevent spread of the virus. The field plot will be 150 ft x 300 ft, including the disposal area.

AGRICULTURAL PRACTICES: Standard agricultural practices will be performed to control insects and pathogens. Aphid populations will be monitored closely and controlled by appropriate insecticides to avoid transmission of virus to control plots. Company representatives will visit the plot three times per week, and monitoring of plants will include observations of morphology, plant vigor, water status, nutrient status, physiological problems, flower initiation, disease problems, insect infestation, and damage from invertebrate and vertebrate pests.

Animals likely to visit the field include the usual fauna (mice, birds).

DATA COUECTION

(1) Fruit count and total weight at each harvest,

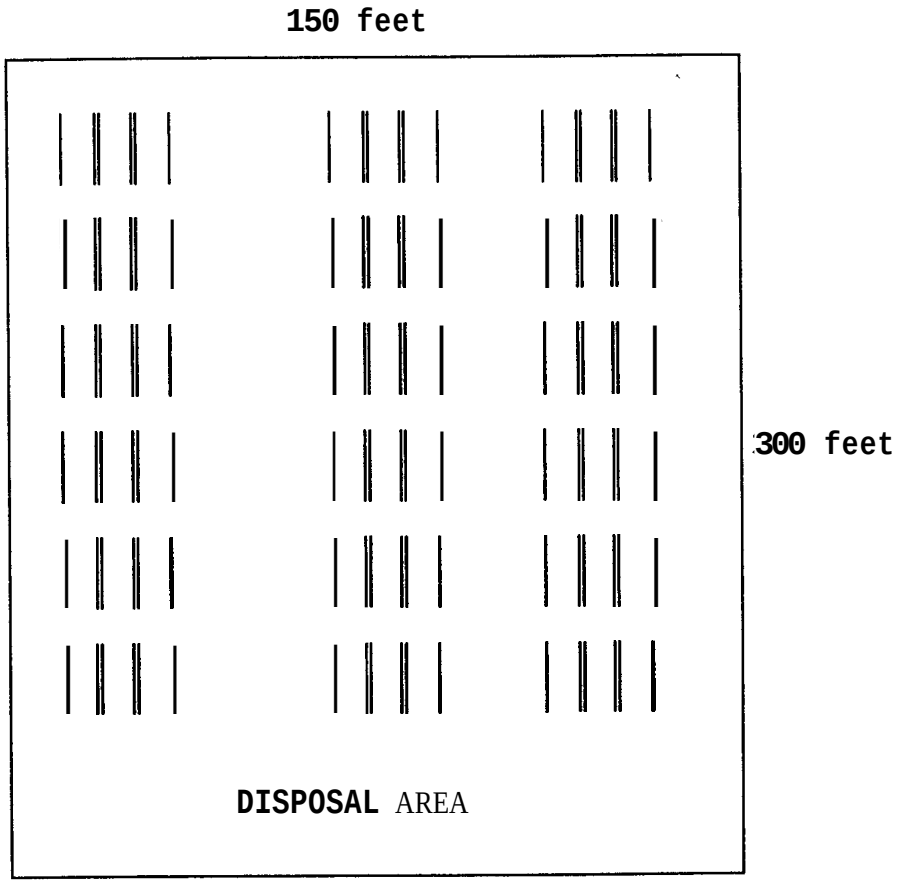
(2) Biochemical and molecular monitoring of virus infection, and

(3) Visual monitoring of symptom development of engineered versus nonengineered plants.

EXPECTED TEST CONCLUSION DATE: September 15.

Initially all field test sites were enclosed by fences and some had more elaborate security measures. These kinds of security are not required but may offer some protection from vandalism. Adequate security may be invisibility from the nearest road or sheltering by surrounding crops (e.g., engineered tomatoes surrounded by border rows of corn). The duration of the field test may be longer than a single growing season; however, in these cases periodic status reports to APHIS are required.

Test Plot Site Design



Single lines - border rows

Double lines - experimental rows

(20 plants per row)

13f. Description of Containment.

Seedlings or seeds of the transformed plants **will** be transported from the greenhouse to the field test location in a van under the supervision of the Paige-Sullivan Biotechnologies personnel who are directly responsible for supervising the field trial. All movements of regulated articles from Halasa Plant Products (North Dakota) to Maryland **will** be under separate permit.

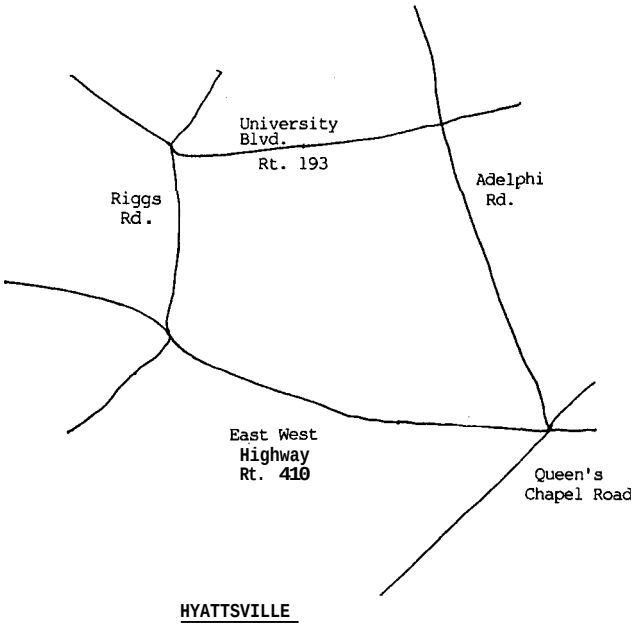
If a regulated article(s) is being moved interstate (prior to or after the field release is initiated), a separate APHIS Form 2000 for movement must be submitted. See Section II for sample movement permit applications.

13g. Description of Containment.

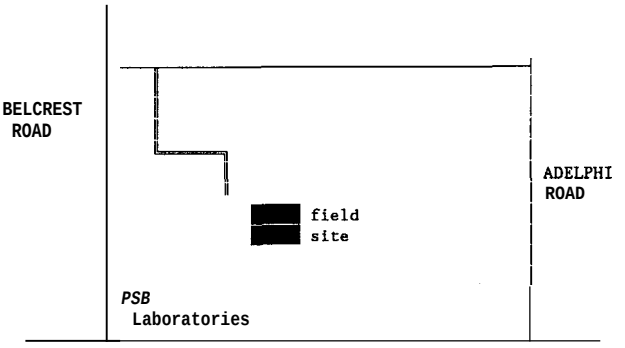
The laboratory, growth chamber facilities, and greenhouses have been inspected and approved by APHIS under previous movement permit applications. Our laboratories meet the NIH Guidelines for Research Involving Recombinant DNA.

Seedlings will be transported directly from the greenhouse to the test location as described in 13f. The experimental field will be located in Prince George's County, Maryland. Map 1 shows the general area of the field trial and map 2 gives greater detail of test location.

Map 1



Map 2



Seedlings should be transported to the field test site in an enclosed vehicle or covered in some acceptable manner if transferred in a truck. Microorganisms should be moved in containers as described in Section IV.

13h. Detailed Description of Containment.

The field test site will be surrounded by a 3-ft-high, chain-link fence. The morphology of Packard Clipper cultivar greatly reduces the chance of cross pollination. Tomato, Lycopersicon esculentum, is a member of the family of plants called Solanaceae. It is a self-pollinating herbaceous perennial that is usually grown as an annual crop in the United States. The natural distribution of wild species of is restricted to the Andean region of South America. As with many self-pollinating species, the flower morphology of tomato greatly facilitates self-pollination (Rick 1976). The pistil is actually enveloped by a solid tube formed by the stamens. When mature, the anthers dehisce and pollen is released by lateral slits into a central cavity. Since the flowers hang down, the pollen moves by gravity towards the mouth of the tube where the stigma is located. Self-pollination then occurs. This cultivar has a much shortened style that places the stigma well within the anther tube, further expediting self-pollination and substantially reducing the opportunity for outcrossing. The Association of Official Seed Certifying Agencies <Anonymous 1971) publishes plant isolation requirements mandated to maintain the purity of seed. The separation distance for foundation tomato seed is 200 ft. No tomato plants will be grown within 660 ft of the test site.

One environmental issue is that the engineered gene could outcross to other populations of Lycopersicon or closely related species in nature. There are no other wild species of Lycopersicon in the United States that could cross pollinate with the experimental plants. Commercial tomato cultivars have not been known to be weedy species. Seeds protected in soil may germinate the following spring (Rick 1976). Therefore, the field test site will be monitored for 6 months following the termination of the experiment for the presence of volunteer tomatoes.

Containment Points To Consider

Could the engineered organism have any impact on: floral communities, faunal communities, endangered or threatened organisms, humans, the health of plants or animals, and genetic resources (e.g., susceptibility of economically important species to herbicides or pesticides) or agricultural production? What are the survival rates of the modified organism in the spectrum of conditions likely to be found in the release area(s) and surrounding environment? What are the organism's reproduction rates in these areas? What is the capability of the organism to disperse from the release area? What are the dispersal mechanisms? What are the consequences of the organism remaining in the environment beyond the planned period? What methods will be used to control or eliminate the organism from the site and the surrounding environment should such action be required? How effective are these methods?

Plants. One of the major concerns for plants is dissemination of the engineered genes by pollen. The Association of Seed certifying Agencies publishes plant isolation requirements for maintaining seed-stock purity. This is a good starting point for designing containment features for many experiments as long as one takes into consideration the percentages of outcrossing assumed in those isolation distances. APHIS recommends that applicants include supporting statements from authoritative persons (e.g., plant breeders or ecologists) stating that the experimental design, location of plot, and local conditions are sufficient to minimize escape of genes to sexually compatible plants. Having considered the pollination characteristics of the species, do wild populations of the species, or related species with which it can interbreed, exist in the vicinity of the field trial or agricultural site? Are any members of the genus of modified plants known to be weeds?

Microorganisms Associated With Plants. Is the organism able to establish itself on/in nontarget species in the surrounding environment? To what extent does the organism survive and reproduce on/in the target plant and/or other plant species in the test site and surrounding environment? Are there any effects on soil microorganisms that are beneficial to plants (e.g., *Rhizobium* and mycorrhizal fungi)? In the case of biological control organisms, can the organism establish itself with nontarget species? Can the modified genetic traits be transmitted to other microorganisms in the environment? What methods are used to monitor the environmental impacts, particularly the population of the modified, target, and nontarget organisms? Can the genetically engineered microorganism be disseminated by wind, water, soil, mobile organisms, or other means?

13i. Final Disposition of Regulated Article:

Fruit and plant material removed from the field for testing in the laboratory will be autoclaved and disposed of as trash or returned to the field for destruction with remaining live plants.

Fruits and plants harvested during the course of the experiment (and not returned to the laboratory) will be buried in the disposal site within the plot for natural decay under compost conditions. All plants and fruits remaining at the termination of the experiment will be treated with the herbicide glyphosate. In greenhouse tests, glyphosate has been shown to Mll both mature transformed and control plants. After the plants have died, the debris will be incorporated into the soil. The test site will be monitored for the next 6 months to make sure all test plants and any volunteers are killed. All tomato plants appearing during this period will be removed either by hand or by another herbicide application, depending on the number of plants involved.

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The following *two* pages are examples of CBI and CBI-deleted text of page III-8 of this Section.

HindII/BamHI fragment of Tn5 which contains NPT 11 (Rao and Rogers 1979). The NOS 3' polyadenylation and termination signal sequences were isolated and fused to NPT II (NEO) gene as previously described (White 1988). The chimeric NPT 11 gene was isolated as a BclII/BamHI fragment and cloned into BglII site of pGV825 to produce pGC831.

DESCRIPTION OF RECIPIENT: The recipient organism, L. esculentum cv. Packard Clipper, is a common commercial cultivar and is a fresh market tomato. It is not widely grown in Maryland because of its susceptibility to CMV. Additional information on the biology of this tomato can be found in section 13h.

DESCRIPTION OF DONOR: CMV has been linked to plant disease in all temperate regions of the world. The virus has an extremely wide host range that includes cereals, forages, woody and herbaceous ornamentals, vegetables, and fruit crops. The RNA of CMV consists of four components of different size (approximate M_r: 1.01, 0.89, 0.68, and 0.33 x 10^6). The three largest RNA's, which are distributed among three separate virion particles, carry all the information needed for successful infection. The genetic information for viral coat protein is carried on RNA 3 and on a coencapsidated subgenomic messenger RNA 4 (Kaper 1984). The viral coat protein assembles around the viral RNA to form the stable virion, which protects the nucleic acid from physical, chemical, or biochemical degradation. The coat proteins from different CMV strains usually have different amino acid sequences that reflect differences in nucleotide sequences of RNA 4 (Kaper 1984).

CMV strain PV 29 (also called strain 1) (American Type Culture Collection Catalogue, 14th Ed., 1988) was propagated as previously described (Lot et al. 1972) and dsRNA isolated as previously described (Diaz-Ruiz and Kaper 1978). Full-length ds cDNA copies of PV 29 CMV RNA 4 were prepared using synthetic oligonucleotide to prime RNA synthesis simultaneously from the 3' ends of both plus and minus strands of denatured ds RNA 4. The primer hybridizing to the plus strand contained an added ClaI recognition site at its 5' end to facilitate forced cloning into the plasmid vector. Additional details of this construct have been published (White 1988), and a reprint appears in appendix 1.

The promoter used in this study was derived from strawberry vein banding virus (SVBV), a caulimovirus (Shepherd 1979). The replication strategy of SVBV is thought to be analogous to the well-characterized cauliflower mosaic virus (CaMV). Upon infection of a cell with SVBV, two major RNA transcripts, designated 40S and 22S (based on their sedimentation coefficients), are produced during the replication of SVBV. These transcripts are analogous to the 35S and 19S CaMV RNAs (Hull and Covey 1983). The 40S SVBV promoter sequences have been characterized (further details in appendix 1). The 40S promoter was isolated as the HindIII/ClaI fragment extending from +9 to -343 with respect to the transcription start site mapped for the 40S RNA. The transcription termination and polyadenylation signal sequences were derived from the nopaline synthase (NOS) gene (Barker et al. 1983). CBI

The [40S promoter] and NOS termination signal sequences were blunt-end ligated to the cloned CMV coat protein gene and inserted into pGV831 at the unique BamH1 site. A map of the resulting plasmid pJLW180 is shown in figure 3. pJLW180 was introduced into the acceptor Ti plasmid pGV2260 by a single homologous recombination, using Sm^R gene of pJLW180 as a selectable marker for cointegration (see fig. 4 for map of cointegrate). The mobilization of pJLW180 from E. coli to Agrobacterium C58C1Rif^R (pGV2260) was performed according to Van Haute et al. (1983). The structure of the T-region was confirmed by Southern blot hybridization.] CBI

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