GENETIC ENGINEERING OF INSECT TOLERANCE IN WALNUT: SUSCEPTIBILITY TO BT.

Abhaya M. Dandekar  Gale H. McGranahan  Pat Vail  
Sandra L. Uratsu  Charles Leslie  Steve Tebbits

ABSTRACT

The goal of this project is to produce transgenic walnut plants resistant to insects through the expression of an insecticidal protein. One such protein is the delta endotoxin (BT) of Bacillus thuringiensis (Bt). We report the preliminary testing of purified toxic fragments obtained from two different strains of Bacillus thuringiensis (HD-1 and HD-73) against three major walnut pests (codling moth, navel orangeworm and Indian meal moth) in control feeding experiments. These tests were successful. We have begun an effort to improve the efficiency of Agrobacterium-mediated transformation of walnut embryos essential for the success of this project. One approach we are investigating is to be able to discriminate transgenic embryos at an early stage. We have used successfully the gene encoding a bacterial β-glucuronidase enzyme as a marker for transformed cells. Our preliminary results are extremely encouraging. Lastly we report our efforts on trying to isolate somatic embryos of the major walnut cultivars (Sunland and Chandler). This is important to introduce this trait into the major walnut cultivar.

OVERALL RESEARCH GOALS AND SCHEDULE

1. Testing of different BT gene products in control feeding experiments with different walnut pests.
2. Transfer and regeneration of transgenic walnut expressing BT.
3. Testing of transgenic embryos and plants.

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<td>of Chandler and Sunland</td>
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<td>Bioassay transformed embryos, seedlings, nuts in lab., field and post harvest</td>
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BACKGROUND

This project aims at providing a solution to the destruction caused by codling-moth, navel orangeworm and Indian meal moth through the creation of new cultivars of walnut that carry a gene(s) providing resistance to these insects through the activity of the BT protein. Thus the project would provide the potential advantage of controlling pests within both the production and post harvest sectors. This proposal also represents a biocontrol approach to this problem that has a genetic basis. Other potential benefits of this research would be through: 1) increased production efficiency associated with lower labor costs that are in turn associated with less frequent spraying of trees; 2) more flexibility that will allow selective control of aphid and mite problems; 3) better post harvest control that will have impact not only on quality issues but may also permit penetration of foreign markets (like Japan) thus increasing revenues through export.

This is a 4 year project and given below are the goals for the first year:

OBJECTIVES OF FIRST YEAR

1. Testing of different BT gene products in control feeding experiments with different walnut pests. (Vail, Tebbits)
2. Improving the efficiency of transformation of walnut somatic embryos with Agrobacterium. (McGranahan, Leslie)
3. Initiating nucellus cultures; somatic embryo formation of commercial cultivars. (McGranahan, Leslie)

The insecticidal activity of Bacillus thuringiensis resides in the spore (Dulmage, 1981) of this bacterium as an inclusion body that contains a crystal (Angus, 1954) of a protein known as the delta-endotoxin (BT). Crystal/spore suspensions of BT have been used commercially for many years in products such as Dipel and Thuricide. The BT toxins are considered an attractive as well as safe biocontrol agent because there has been no known effect on non-target organisms for the 20 years they have been in commercial use. Although different strains of Bacillus thuringiensis make toxins that are active against different groups of insects, the relationships between structure and function of the various toxins is presently the subject of intense research in several laboratories worldwide. One important byproduct of this effort is the isolation, characterization and DNA sequence analysis of several genes encoding BT effective against Lepidopteran insects (Schnepf et al. 1985, Shibano et al. 1985, Adang et al. 1985, Thorne et al. 1986, Wabiko et al. 1986, Hofte et al. 1986, and Geiser et al. 1986). Very recently this information was put to good use when transgenic plants expressing the BT toxin were obtained in tobacco (Vaeck et al. 1987) and tomato (Fischhoff et al. 1987). These transgenic plants expressed sufficient quantities of BT toxin to protect the plants from damage caused by the feeding larvae of tobacco or tomato hornworm, both voracious production pests. The focus of our effort is to distill this body of knowledge to produce something that is of use for walnuts.

Toxins produced by strains HD-1 and HD-73 of Bacillus thuringiensis were used in our study and were chosen for the following two reasons i) both displayed (in the hands of other research workers) a high degree of toxicity towards a wide diversity of lepidoptera species. ii) The genes encoding the active toxin species have been cloned facilitating the construction of vectors for transfer of these genes to plants.

2. Improving the efficiency of transformation of walnut somatic embryos with
We have developed and recently reported a gene transfer system for walnuts (McGranahan et al., 1988). That report describes the methods we used to transfer a marker gene for kanamycin resistance from a disarmed strain of *A. tumefaciens* to embryogenic cells of a walnut somatic embryo. These transformed cells then gave rise to new embryos which were multiplied, screened and germinated. Two transgenic walnut clones resulted from this work.

Our recent objective has been to improve the efficiency of transformation and selection in the system by inserting an additional selectable marker gene which could be detected earlier and more reliably than kanamycin resistance. We used the bacterial beta-glucuronidase (GUS) gene in two different chimeric constructs contained in the vectors pCGN 7001 (Fig. 1) and pCGN 7314 (Fig. 2). In addition to the GUS genes these vectors also contained the gene for resistance to kanamycin. Specific goals were to determine: a) if the GUS gene product was expressed and could be reliably detected in transformed walnut tissues; b) if selection for GUS expression could be initiated earlier and more efficiently; c) if GUS could be used to determine which parts of embryos were most susceptible to transformation and if these overlapped areas that are most embryogenic such as the radicle. d) if kanamycin selection could be applied during early development without inhibiting transformant embryo formation; e) if kanamycin selection enhanced transformant recovery by suppressing competing non-transformant embryos.

3. **Initiating nucellus cultures; somatic embryo formation in commercial cultivars.**

The gene transfer system we developed for walnuts relies on repetitive somatic embryogenesis to regenerate embryos and plants containing a transferred gene i.e., transgenic plants (McGranahan et al., 1988). The embryogenic tissues we used to develop the system were originally obtained from the cotyledons of developing zygotic embryos and therefore were not genetically identical to the parent cultivar. For our system to be applied to genetic improvement of walnut cultivars it is important that we have the means to insert a desired gene into a cultivar of choice and not into its offspring. One means of achieving this goal is to initiate repetitively embryogenic cultures directly from the cultivar of choice. In other crops, nucellus has been used to initiate embryogenic cultures (George and Sherrington, 1984).

Our objective was to determine if embryogenic cultures could be initiated from nucellus of the walnut cultivars Sunland and Chandler.

**PROCEDURES**

1. **Testing of different BT gene products in control feeding experiments with different walnut pests.** The toxins produced by strains HD-1 and HD-73 of *Bacillus thuringiensis* were chosen for this study. The active component of the toxin protein is the N-terminal 1/3 of the protein. Purified active toxin fragments of the BT toxins HD-1 and HD-73 were obtained from Dave Fischoff at Monsanto. The active toxin fragments are obtained after tryptic digestion (with the protease Trypsin) of the purified toxin followed by separation by column chromatography. Purified toxin is obtained by solubilization of pure crystals of HD-1 or HD-73 or by over expression of the genes in E.coli. The purified toxin fragments were stored in 100 mM Sodium Carbonate (pH 10.0), 50 % Glycerol and 10 mM DTT (dithiothreitol) frozen at -80°C. Dilutions were made in 100 mM Sodium Carbonate buffer (pH 10.0) containing 10 mM DTT. Two controls were used plain buffer and buffer with DTT to ensure that the buffer or DTT were not toxic to the insects.

The test insects CM (codling moth), NOW (navel orange worm and IMM (Indian meal moth) were reared and the bioassays conducted at the Horticultural Research Institute at Fresno, California. The susceptibility of each of these insect species to the Bt toxins HD-1 and HD-73 was determined.
by feeding (per os) experiments. Serial dilutions of each protein were layered onto the surface of an agar-based diet (Bioserve #9370) and the dose was expressed as micrograms per sq. mm of diet surface. One neonatal larva was placed in each of 20 vials (containing ca. one ml of surface inoculated diet) for each insect species and dilution. The larvae were then incubated at 80°F and mortality (dose response) evaluations were made daily. The test was replicated twice to provide a total of 40 neonatal larvae tested to determine the time (LT) or dose (LD) required to reach 50 or 95% mortality (Table 1, Table 2).

2. **Improving the efficiency of transformation of walnut somatic embryos with Agrobacterium.** The chosen vectors contain two chimeric genes that encode beta-glucuronidase (gus) and antibiotic resistance to kanamycin. We have used two different vectors kindly provided by L. Comai (Calgene). The structure of the chimeric genes in these two vectors is as follows: pCGN 7001 (binary) CaMV 35S/kan/tr7, mas/gus/mas (Fig. 1); pCGN 7314 (binary) (CaMV 35S)x2/gus/mas, mas/kan/mas (Fig. 2). These vectors were introduced into the Agrobacterium host EHA101 (Hood et al., 1986) by a triparental mating procedure using a helper plasmid pRK2073 (Ditta et al., 1980). EHA101 was used as the control.

The Agrobacterium strains were grown at 26-28° C in 523 medium (Rodriguez and Tait, 1983) overnight and then removed by centrifugation (5000xg, 10 min.) and resuspended to a density of 2.5 x 10^8 cells/ml in DKW medium containing 100 µM acetosyringone (AS). These resuspended cells were used as inoculum.

Each Agrobacterium strain (EHA101-control, pCGN 7001, pCGN 7314) was used to inoculate 40 small (2-5 mm) white embryos of a repetitively embryogenic culture line (SU-2) derived from the immature cotyledon of an open pollinated 'Sunland' seed. Embryos were soaked for 10-15 minutes in the inoculum, blotted lightly on sterile filter paper and plated on solid basal DKW media (Driver and Kuniyuki, 1984) containing 100 µM acetosyringone. After 48 hours, embryos were rinsed in basal DKW containing 500 mg/l cefotaxime and transferred to plates of the same medium solidified with Gelrite. After 24 hours half of the embryos in each treatment were transferred to medium containing both cefotaxime (500 mg/l) and kanamycin (100 mg/l) resulting in 6 different treatments of 20 embryos each. Embryos in each treatment were transferred to fresh medium every 7 - 10 days.

Four of the original embryos (Eo generation) in each treatment were sacrificed for evaluation of GUS expression after one and 2 1/2 weeks. The remaining Eo embryos were maintained and secondary embryos (E1) were removed from them for evaluation beginning at week 6. Embryos that tested positive were multiplied as subclones for retesting.

Expression of the GUS gene was tested by an X-Gluc (5-bromo-4-chloro-3 indolyl glucuronide) histochemical assay based on the method of Jefferson (1987). X-Gluc is a substrate which produces a localized blue precipitate in cells expressing the GUS gene. X-Gluc substrate solution was prepared by dissolving X-Gluc (Clontech) to a 0.3% w/v solution in dimethylformamide. This was diluted to 1 mM X-Gluc with 100 mM sodium phosphate buffer (pH 7.0) containing 0.006% Triton-X 100 and 0.5 mM KFe Cyanide. This solution could be kept refrigerated for at least two weeks.

E1 embryos were tested for transformation by cutting a small piece from each embryo and immersing it in X-Gluc solution at room temperature or 37°C. Embryos were observed for blue color at intervals beginning at 10 minutes after immersion up to 24 hr. If the piece developed a distinct blue color the corresponding embryo was kept and multiplied as a subclone. Later these subclones will be further analysed for the presence of integrated T-DNA following which they will be germinated for the production of transgenic plants.
3. **Initiating nucellus cultures; somatic embryo formation of commercial cultivars.** Pollen isolation bags were placed over immature flowers on walnut cvs. Sunland (70 bags) and Chandler (60 bags). At peak fertility pollen was injected into half of the bags of each cultivar; the flowers in the remaining bags were not exposed to pollen. 'Sharkey' was used as the pollen source for 'Chandler' because it has a unique isozyme banding pattern (Arulsekar, pers. com.) which would allow differentiation between cultures initiated from zygotic and maternal tissues. Because 'Sharkey' pollen could not be obtained in time for 'Sunland' pollination, 'Payne' pollen was used instead, even though it does not have as unique a pattern.

Two to three weeks after pollination, developing nutlets, 7-12 mm in diameter were collected, surface-sterilized (80% Ethanol dip, followed by 20 min. in 20% Clorox bleach), rinsed and dissected. Either the entire ovule or the nucellus alone was then cultured on standard initiation medium (Tulecke and McGranahan, 1985) or a modified initiation medium with half-strength salts or with 2,4-D (1.0 mg/l) instead of standard hormones. Cultures were maintained on a 2-3 wk transfer interval throughout the experiment. After one to two months, cultures were removed from initiation medium and placed on basal DKW media (Driver and Kuniyuki, 1984). Modifications of the basal DKW medium were also used; these included varying levels of sugars and salts. Embryos that developed were multiplied and the mobility of a selected group of isoenzyme markers were analyzed by S. Arulsekar.

**RESULTS AND CONCLUSIONS**

1. **Testing of different BT gene products in control feeding experiments with different walnut pests.** The data shown in the attached tables 1 and 2 and in figures 3-8 demonstrate that both proteins are toxic to the target insects at relatively low concentrations (doses are expressed in micrograms of protein per sq mm of diet). In general, HD-1 tended to be more toxic to CM and NOW as compared to HD-73. IMM was the most susceptible species and NOW the least susceptible species to either toxin. Furthermore, surface-inoculated diet that had been at 80° F for eight (8) days showed no loss of activity when infested with neonatal larvae, indicating that the BT proteins remained active during the duration of the study.

Results from other tests have indicated that all three species will survive and develop on an agar-based diet made from only freeze-dried somatic walnut embryos and containing an antibiotic to inhibit bacterial growth. Future tests are planned to determine the effectiveness of a protein incorporation technique where the BT protein will be mixed with and distributed throughout the Bioserve agar diet. Although the surface inoculation technique described above is quicker and simpler, and requires less protein to run, the incorporation technique will more accurately simulate conditions that will be encountered by the insect eating transgenic plant material.

2. **Improving the efficiency of transformation of walnut somatic embryos with Agrobacterium.** We were successful in inserting and detecting expression of the GUS gene in walnut embryos. GUS-positive embryos were easily detected within 3 hours of exposure to the X-Gluc substrate. The distinct blue color first could be observed on cut surfaces or areas damaged in handling. With longer periods of exposure the blue color spread internally and frequently developed at the cotyledon tips where the tissue is very thin. In most cases the blue color spread uniformly but in one case only the veins showed color even after 48 hours suggesting a tissue specific transformation. The blue from the transformed plant could be distinguished from that caused by the bacteria if present. Bacterial blue was diffuse on the embryo surface, tended to be a lighter shade and became apparent after a longer exposure. When bacteria were present the X-Gluc solution itself became cloudy and blue over a 24 hour period; the solution surrounding clean embryos remained clear.

E0 embryos tested in X-Gluc at one week still had abundant bacteria on their surfaces which
interfered with any possibility of detecting transformed cells. The cefotaxime rinse before plating was designed to reduce this problem but was unsuccessful.

E0 embryos from inoculation with pCGN 7001 tested at 2 1/2 weeks had numerous small (0.05 mm), superficial spots of blue on their hypocotyls after 3 hours in X-Gluc. Embryos which had not been exposed to kanamycin clearly had more spots (mean >200 spots/embryo hypocotyl) than embryos exposed to kanamycin (mean = 24 spots/embryo hypocotyl). The cotyledons had fewer spots than the hypocotyls (mean = 4 with kanamycin, 2 no kanamycin). Embryos inoculated with EHA101 had no spots and only one embryo inoculated with pCGN 7314 had any spots. Spots in all cases frequently appeared to be associated with sloughy cells and hairlike structures and may have no bearing on future transformation potential.

Testing of E1 embryos began at 5 1/2 weeks after inoculation and continued at 1 to 2 week intervals through week 16. Eight E0 embryos produced 186 E1 embryos that were GUS positive (Table 3). Three of these E0 embryos had been exposed to pCGN 7314 plus kanamycin, 4 to pCGN 7001 plus kanamycin, and one to pCGN 7001 without kanamycin. No embryos inoculated with the control EHA101 produced GUS positive embryos. Kanamycin exposure enhanced transgenic embryo production; 21.2% of the exposed embryos produced transgenic embryos, whereas only 3.4% of the unexposed embryos did. In this study it was not possible to determine how many independent insertions occurred in each E0 embryo because more than one embryo could have developed from an insertion site. We suspect this is the case because of the increasing percent of transformed embryos detected at each harvest (Table 4). The frequency of transformation events will be clarified by DNA analysis.

In summary, the GUS marker gene provided an early, reliable screen for selecting transgenic embryos. Early exposure to kanamycin increased the percent of embryos producing new transgenic embryos. We recommend that all future vectors used for walnut transformation contain these two markers.

3. Initiating nucellus cultures; somatic embryo formation of commercial cultivars. Five of the 148 nutlets cultured produced embryogenic culture lines; two were derived from the ovules of unpollinated 'Sunland' nutlets and three from pollinated ovules. The latter were analyzed by electrophoresis and zygotic parentage was confirmed in two lines; although it could not be confirmed we are assuming the third line is also of zygotic origin. Insufficient culture material was available for testing the two lines derived from unpollinated ovules. An additional 65 cultures derived from pollinated and unpollinated ovules and nucellus of both 'Sunland' and 'Chandler' are still being maintained; eight have formed callus but none of have produced embryos yet.

We still consider it essential to develop the means to initiate repetitively embryogenic cultures from the cultivars themselves because the gene transfer system we developed is still the most promising for woody plants like walnuts which have a long generation interval. The system has a built-in means to select out chimeras which could be a problem with other systems such as the shoot apex system. We plan to continue this work for an additional year with modifications designed to induce apomixis and somatic embryo induction from apomictic embryo cotyledons.

REFERENCES


Schnepf HE, HC Wong and HR Whiteley. 1985. The amino acid sequence of a crystal protein from Bacillus thuringiensis deduced from the DNA base sequence. J. Biol. Chem. 260: 6264-6272.


Table 1

Estimated lethal times (LT$_{50}$, LT$_{95}$) for neonate larvae of 3 lepidopterous insects exposed to *B. thuringiensis* proteins (HD-1 and HD-73) per os using a surface inoculation technique on agar diet.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Insect</th>
<th>Dose ($\mu$g/sq. mm)</th>
<th>LT$_{50}$ (days)</th>
<th>LT$_{95}$ (days)</th>
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<td>HD-1</td>
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<td>0.01</td>
<td>2.17</td>
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<td>3.44</td>
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<td></td>
<td>navel orangeworm</td>
<td>0.07</td>
<td>3.98</td>
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<td>0.01</td>
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<tr>
<td></td>
<td>Indianmeal moth</td>
<td>0.07</td>
<td>----</td>
<td>----</td>
</tr>
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<td></td>
<td></td>
<td>0.01</td>
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<td></td>
<td></td>
<td>0.001</td>
<td>0.96$^1$</td>
<td>1.94$^1$</td>
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<tr>
<td>HD-73</td>
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<td>0.1</td>
<td>1.83</td>
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<td></td>
<td>Indianmeal moth</td>
<td>0.1</td>
<td>----</td>
<td>----</td>
</tr>
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<td></td>
<td></td>
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<td>0.94$^1$</td>
<td>1.93$^1$</td>
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<td></td>
<td></td>
<td>0.001</td>
<td>0.97</td>
<td>2.35</td>
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</table>

$^1$Nonsignificant regression (p>0.05), but serves as an estimate.
Table 2

Estimated lethal doses (LD$_{50}$, LD$_{95}$) for neonate larvae of 3 lepidopterous insects exposed to *B. thuringiensis* proteins (HD-1 and HD-73) per os using a surface inoculation technique on agar diet.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Insect</th>
<th>Day #</th>
<th>LD$_{50}$ (µg/sq. mm)</th>
<th>LD$_{95}$ (µg/sq. mm)</th>
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<td>navel orangeworm</td>
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<td>0.012$^1$</td>
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<td></td>
<td></td>
<td>3</td>
<td>0.000021$^1$</td>
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<td>HD-73</td>
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<td>0.0036</td>
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<td>navel orangeworm</td>
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<td>10.01$^1$</td>
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<td></td>
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<td>8</td>
<td>0.018</td>
<td>0.84</td>
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<td></td>
<td>Indianmeal moth</td>
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<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.0000096</td>
<td>0.0011</td>
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$^1$Nonsignificant regression ($p>0.05$), but serves as an estimate.
### TABLE 3. Frequency of GUS-positive (GUS+) embryos.

<table>
<thead>
<tr>
<th>Inoculum medium</th>
<th>E₀ embryos producing Kanamycin</th>
<th>GUS+ E₁ embryos (number) (%)</th>
<th>E₁ embryos producing GUS+ E₁ embryos (number) (%)</th>
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<tbody>
<tr>
<td>pCGN 7314 +</td>
<td>16</td>
<td>3 (19%)</td>
<td>403 (130%)</td>
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<tr>
<td></td>
<td>16</td>
<td>0 (0%)</td>
<td>299 (0%)</td>
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<td></td>
<td>32</td>
<td>3 (9%)</td>
<td>702 (130%)</td>
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<td>pCGN 7001 +</td>
<td>17</td>
<td>4 (24%)</td>
<td>331 (53%)</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>1 (8%)</td>
<td>416 (3%)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5 (17%)</td>
<td>747 (56%)</td>
</tr>
<tr>
<td>EHA101</td>
<td>3</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0 (0%)</td>
<td>654 (0%)</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0 (0%)</td>
<td>654 (0%)</td>
</tr>
</tbody>
</table>

### TABLE 4. Frequency of GUS-positive E₁ embryos from time of inoculation.

<table>
<thead>
<tr>
<th>E₀ Embryo</th>
<th>Kanamycin</th>
<th>Weeks from inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>1 pCGN 7314 +</td>
<td>1/4 (25)²</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>43 pCGN 7314 +</td>
<td>0/20 (0)</td>
<td>---</td>
</tr>
<tr>
<td>47 pCGN 7314 +</td>
<td>1/40 (3)</td>
<td>---</td>
</tr>
<tr>
<td>9 pCGN 7001 +</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>11 pCGN 7001 +</td>
<td>---</td>
<td>0/25 (0)</td>
</tr>
<tr>
<td>32 pCGN 7001 +</td>
<td>0/13 (0)</td>
<td>---</td>
</tr>
<tr>
<td>36 pCGN 7001 +</td>
<td>3/14 (21)</td>
<td>13/16 (81)</td>
</tr>
<tr>
<td>26 pCGN 7001 -</td>
<td>0/1 (0)</td>
<td>1/9 (11)</td>
</tr>
</tbody>
</table>

² GUS-positive E₁ embryos/Total E₁ embryos harvested, (percent GUS-positive embryos)
Figure 1

pCGN7001

~30 kb

Eco RI 80 m HI
Eco RI 8 am HI

PVCK 102

LB GENT

35S-5' Tr 7-3'

mas 5'

mas 3'

kan GUS

RB

30/0
Figure 2
Fig. 3
Codling Moth vs. HD-1 Bt Protein
(Dosages in micrograms per sq. mm)

% Mortality

# Days Postinoculation

--- 0.07
--- 0.01
--- 0.001
--- 0.0001
--- 0.00001
--- CONTROL

Fig. 4
Codling Moth vs. HD-73 Bt Protein
(Dosages in micrograms per sq. mm)

% Mortality

# Days Postinoculation

--- 0.1
--- 0.01
--- 0.001
--- 0.0001
--- 0.00001
--- CONTROL
Fig. 5  
Navel Orangeworm vs. HD-1 Bt Protein  
(Dosages in micrograms per sq. mm)

Fig. 6  
Navel Orangeworm vs. HD-73 Bt Protein  
(Dosages in micrograms per sq. mm)
**Fig. 7** Indianmeal Moth vs. HD-1 Bt Protein

(Dosages in micrograms per sq. mm)

![Graph showing mortality of Indianmeal Moth vs. HD-1 Bt Protein](image)

**Fig. 8** Indianmeal moth vs. HD-73 Bt Protein

(Dosages in micrograms per sq. mm)

![Graph showing mortality of Indianmeal Moth vs. HD-73 Bt Protein](image)