



Genetic Approach to Identifying Bt Resistance Genes In *Heliothis virescens*

Joan LeGloahec and Linda J. Gahan

A gardener at Colonial Williamsburg picks tobacco budworm larvae from his crops as he explains to visitors that these insects have been eating plants since the early days. As farmers around the world have battled voracious insect pests, the agrochemical industry has come to their aid with various insecticides. Spraying crops with these chemicals has worked to kill the invaders and increase crop production until the insects develop resistance. When this happens farmers switch to another chemical to rid their fields of these pests. Meanwhile the environmentalists are concerned about the effects of insecticides on other wildlife and humans.

In the 1990's the agricultural industry took a new approach by creating transgenic crops like Bt cotton and Bt corn. The *Bacillus thuringiensis* bacteria have genes that produce proteins toxic to these insect pests. Specific Bt genes have been genetically engineered into various crops so the plants make enough Bt toxin to kill insects that feed on them. The question remains: Will insects eventually develop resistance to Bt toxin?

The EPA recommends a "high-refuge" strategy that requires farmers growing Bt cotton to also grow adjacent fields of non-Bt cotton. Insects feeding on normal cotton will not be pressured to develop resistance and will mate with insects from Bt cotton fields. If resistance has emerged in the Bt fields, the offspring of these matings are unlikely to produce resistant progeny since the mutation is recessive. The development of Bt resistance is also the interest of scientists who want to assist farmers with pest management. Knowing which genes have been changed in Bt resistant insects will enable the agricultural industry and governmental agencies to devise new strategies to maintain the efficacy of transgenic crops.

Our research group uses a genetic approach to identify and characterize Bt resistance genes. Since there is no Bt resistance in field populations of *Heliothis virescens* (*Hv*), we study a Bt-resistant laboratory strain called YHD2, developed by Fred Gould at North Carolina State University. *Hv* collected from the field were reared on a diet containing very low doses of Bt toxin Cry1Ac. Individuals that survived this treatment were mated, and offspring were selected on the toxin. This mating and selection process yielded a YHD2 strain that is 10,000 times more resistant to Cry1Ac than the susceptible strain. This highly resistant YHD2 strain has multiple Bt resistance genes; these genes are fixed, and resistance is recessive.

Our genetic approach uses two crosses prepared as single-pair matings. The first cross is between YHD2 females and susceptible males to yield F1 families. Individuals from the F1 families, selected on low dose Cry1Ac diet for seven days, grow slowly because of the recessive nature of Bt resistance. A second backcross is constructed between F1 and YHD2 individuals, with offspring selected on toxin in a growth inhibition assay. Backcross families showing a bimodal distribution in size after seven days on toxin are analyzed for resistance. Individuals from female informative families are used to map the resistance gene on a particular linkage group, since there is no crossing-over during meiosis when the female backcross parent is the F1. The order of DNA markers on a particular linkage group can be determined using individuals from a male informative backcross family, since crossing-over occurs in the F1 male. Southern blots are prepared using DNA from small and large offspring from female and male informative backcross families plus parents and grandparents. DNA markers already assigned to the 31 linkage groups are used as probes to identify the linkage group that contains the Bt resistance gene, and then to order DNA markers on that linkage group. The goal is to find DNA markers on either side of the Bt resistance gene close enough to positionally clone the resistance gene. Success of this approach depends on the availability of DNA markers on each of the 31 linkage groups and polymorphism for these markers in the resistant and susceptible strains.

Our approach to identify genes involved in Bt resistance mechanisms utilizes candidate genes. Cadherins and N-aminopeptidases bind Bt toxins, midgut proteases activate Bt toxins, alkaline phosphatases may function as oligomeric toxin receptors, and N-acetylglycosamine transferases glycosylate GPI-anchored midgut membrane receptor proteins. Our research shows that the cadherin-like gene on linkage group 9 (LG9) in YHD2 has a retrotransposon inserted in the fifth cadherin repeat (**Fig. 1**)¹. As a result the cadherin-like protein translated from this mutant gene (r1) is truncated and is never deposited as an integral protein in the midgut membrane, where it would normally bind the activated Bt toxin. Examining two cDNA libraries, one constructed from the YHD2 Bt resistant strain and one made from the susceptible *Hv* strain, led to this discovery. By designing a number of degenerate primers to the cadherin-like gene whose protein product is known to bind Bt toxin in *Manduca sexta*, gene fragments were PCR amplified and used to probe these two libraries. Sequencing of positive clones from the libraries revealed a large foreign DNA insertion in the cadherin-like gene from YHD2. In addition the size of the messenger RNA for the cadherin-like gene was larger in the Bt resistant YHD2 strain compared to the susceptible strain. Further studies by Juan Luis Jurat-Fuentes showed the r1 mutant from YHD2 does not



express the full-length cadherin-like protein.²

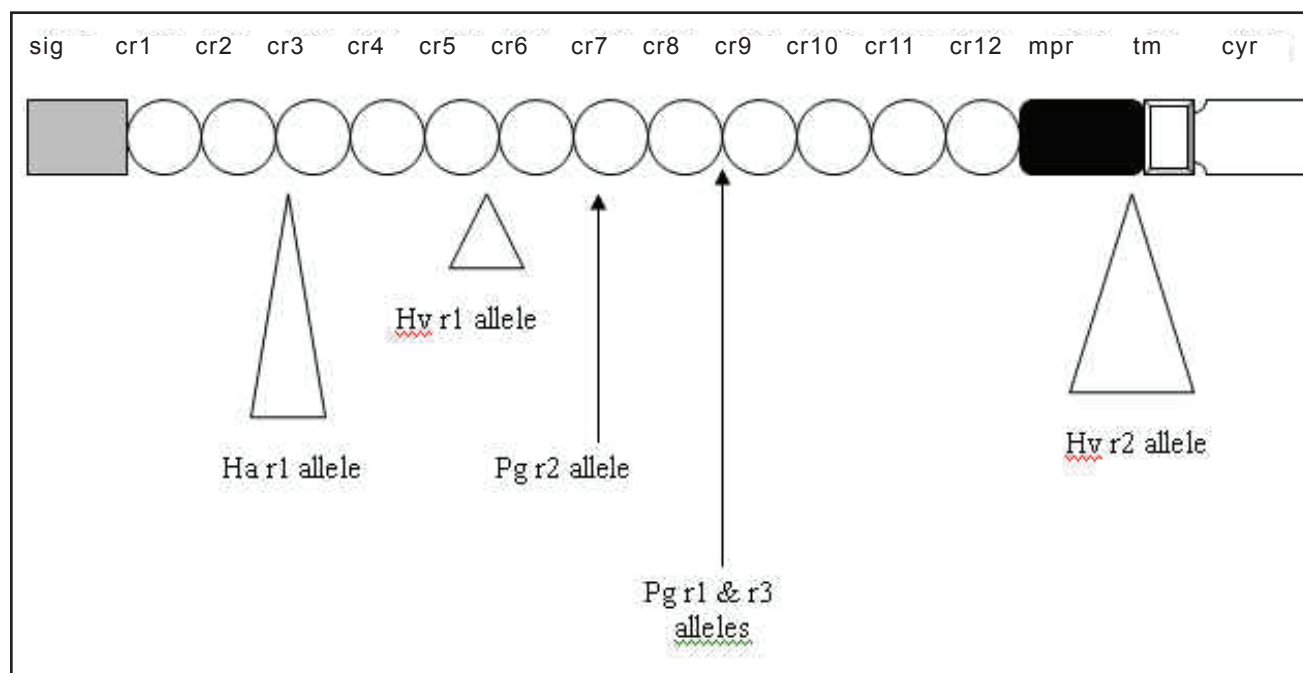


Figure 1. The position of mutations in the cadherin-like gene found in *Heliothis virescens* (r1 and r2) and in *Pectinophora gossypiella* (r1, r2, r3) and *Helicoverpa armigera* (r1). Regions of the cadherin-like gene are shown: sig, signal sequence for protein positioning; cr, cadherin repeat; mpr, membrane proximal region; tm, transmembrane region; cyr, cytosolic domain.

In the pink bollworm, *Pectinophora gossypiella*, Morin et al. located three independent mutations in the cadherin-like gene that conferred Bt resistance to insects found in field populations of Bt cotton³. Xu et al. discovered a stop codon in the cadherin gene sequence of a GYBT Bt resistance strain of *Helicoverpa armigera*.⁴ In a study to determine the frequency of resistance to Cry1Ac in field populations of *Hv*, Fred Gould set up 2000 matings of *Hv* field-collected males with YHD2 females. The offspring from each mating were selected on Bt toxin. Any mating that produced offspring, half of which would grow on Bt toxin, was considered to contain a Bt resistant allele in the field-collected male parent. The study found three such males in field populations of *Hv*, which were frozen for later analysis.⁵ Years later when the cadherin-like gene had been implicated in a Bt resistance mechanism, our laboratory sequenced the gene from one of the frozen, field-collected males. This male contains two alleles for the cadherin-like gene, one similar to the susceptible and one containing a large insertion of another type (r2) in the membrane-proximal region of the gene. Offspring from the cross between this male (r2,s) and the YHD2 female (r1/r1) can grow on Bt toxin if they received the r2 allele from their father and the r1 allele from their mother (unpublished results). Thus, evidence is mounting that the cadherin-like gene is involved in resistance to Bt toxin in Lepidoptera.

The r1 mutation in the cadherin gene accounts for up to 80% of Bt resistance in YHD2. This fact and the analysis of other crosses suggest multiple Bt resistance genes exist in this strain. In order to study these genes, it was necessary to separate the cadherin-like r1 gene from the rest of the Bt resistance genes. The idea was to create a YEE strain lacking the cadherin r1 mutation, a YFO strain containing only the cadherin r1 mutation, and a reconstituted YHD3 strain having all the Bt resistance genes. To construct these three strains, a YHD2 female was mated to a susceptible male, and F1 offspring were selected on a low dose Cry1Ac toxin diet. F1 females were backcrossed to YHD2 males and the BC offspring were selected on Bt toxin for seven days, weighed, and transferred to normal diet to complete development. The smallest individuals from the BC were used to create the YEE strain. The presence of the r1 mutation in the cadherin gene can be determined by PCR using forward and reverse primers designed around the insertion site of the retrotransposon in the cadherin gene. Matings were set up between individuals from YEE families, offspring were selected on Bt toxin, and the parents of these matings were analyzed for the presence of the r1 mutation. Offspring from parents lacking the r1 mutation and growing well on toxin were used to create the next generation of the YEE strain. This process continued until no r1



mutation was observed. Matings and selection on toxin have continued for 58 generations to fix the Bt resistant gene(s). To create YFO, BC individuals of medium size were mated with susceptible individuals, offspring were selected on a low dose of Bt toxin and the YFO parent of these crosses was tested for the presence of the r1 mutation. This process of mating YFO resistant to susceptible individuals was continued for a total of six generations in order to remove other Bt resistance genes. After this, YFO individuals were mated and selected on toxin, and parents were analyzed for presence of the r1 allele. Offspring from families whose parents contained the largest number of r1 alleles were used in constructing the next generation. Matings continued until the r1 allele was fixed in YFO and then to maintain the strain. Mating the largest BC offspring, selecting for maximal growth on Bt toxin, and gradually increasing the toxin concentration for 58 generations created YHD3. In addition three analogous strains were created in like manner and designated RER (no cadherin r1), RFO (only cadherin r1) and REE (all resistant genes).

Analysis of the Bt resistance genes in these strains has been accomplished in two ways. Individuals of a resistant strain are crossed to the susceptible individuals to create F1 families, then backcrosses are set up between F1 and the resistant individuals. Offspring from both crosses are selected for resistance on Cry1Ac. As explained earlier, BC families in which the F1 parent is a female and her offspring exhibit a bimodal distribution of weights after toxin treatment can be used to map the resistance gene. BC families where the F1 parent is a male are used to order DNA markers on the linkage group containing the resistant gene. The second way of analyzing resistance is to cross two resistant strains to form the F1 and then backcross F1 to the most resistant strain.

Using this genetic approach, we have identified a resistance gene on LG2 and have closely ordered DNA markers on either side of it. The initial cross between YFO and YHD3 followed by a backcross to YHD3 gave us this result. An initial cross between RER and the susceptible strain also confirmed this fact. YEE appears to have two resistance genes. Crosses between resistant strains are being constructed to analyze the other gene, possibly on LG10. A *Hv* Bac library provides an excellent resource tool for walking to the resistance gene from closely proximal DNA markers. The recently sequenced *Bombyx mori* genome assists our efforts to find genes closely linked to Bt resistance, as our use of this genetic approach moves forward to identify Bt resistance genes.

References

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Joan LeGloahec and Linda J. Gahan
Clemson University
Department of Biological Sciences
glinda@clemson.edu