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Potential Strategies for Prolonging the Usefulness of *Bacillus thuringiensis* in Engineered Rice

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ABSTRACT A laboratory bioassay that incorporates *Bacillus thuringiensis (Bt)* purified crystal protein toxins into an artificial diet has identified three toxins, CryIA(b), CryIA(c), and CryIIA, to be effective against the yellow stemborer, *Scirpophaga incertulas* (Walker). Research is aimed at engineering rice that incorporates genes of one of or more of these toxins so as to mimic the insecticidal action of the insect Pathogen. Because the yellow stemborer is monophagous on rice, a concern is that homogeneous plantings of the transgenic rice could accelerate adaptation of the insect to *Bt*. The paper discusses potential strategies for slowing the rate of adaptation that include the use of multiple *Bt* toxins, promoters that express the toxins only in specific plant tissues at specific times, and mixing transgenic and non-transgenic plants.

KEY WORDS *Bacillus thuringiensis*, rice, engineered rice, yellow stemborer, durable crop protection, insect resistance

Many public and private organizations are interested in producing engineered rice that mimics the insecticidal action of the insect pathogen *Bacillus thuringiensis* (*Bt*). Genetic engineering of both japonica and indica varieties of rice is now possible through direct uptake of DNA into chromosomes of protoplasts and regeneration of these protoplasts back into mature plants, as reviewed by Hodges et al. (1991). One or more laboratories already have incorporated the *Bt* genes into japonica rice. As early as 1987, genetic engineers had moved the gene codes for *Bt* toxins into tomato, tobacco, and potato (Vaeck et al. 1987).

Under natural conditions, *Bt*'s heat resistant spores protect the integrity of the bacterium's DNA for long periods of time until conditions are favorable for germination and regeneration. During sporulation, *Bt* produces cryatal proteins that contain the insecticidal toxins. Upon ingestion by an insect, the crystals (insoluble in water at neutral pH) solubilize under the alkaline conditions of the insect's midgut and release delta-endotoxins (Cry proteins). Midgut proteases activate the delta-endotoxins. Activated toxins interact with receptors of the larval midgut epithelium disrupting membrane integrity and, ultimately, insect feeding and digestion. Different strains of *Bt* produce distinct toxins, and individual strains may produce a number of different toxins.

Gill et al. (1992) recently reviewed of the mode of action of the *Bt* toxins. Several species of crops containing the *Bt* delta-endotoxin genes currently are being evaluated for field performance (Fuchs 1991). Because *Bt* is relatively non-toxic to humans, most beneficial insects, and other nontarget organisms, it does not present the serious environmental and safety concern that chemical insecticides often do (Flexner et al. 1986).

However, experience with *Bt* as a crop spray has given rise to concerns about eventual insect pest resistance to the *Bt* toxins. Field populations of the diamondback moth, *Plutella xylostella* (*L.*), a major insect pest of vegetables, adapted to and could no longer be controlled effectively by *Bt* foliar sprays in some areas when it was used intensively(Tabashnik et al. 1990, Shelton & Wyman 1991).

An effective *Bt* trsansgenic rice deployment system for controlling the yellow stemborer, *Scirpophaga incertulas*(Walker)(Lepidoptera: Pyralidae), would have considerable potential. Widely spread in rice in tropical Asia, the pest can cause substantial crop losses (Khan et al. 1991). Existing rice cultivars lack dependable sources of resistance to the insect. Chemical control is often ineffective because of its boring habit. Newly emerged larvae (usually less than 24 hr old) bore inside the rice plant tissues where they escape the foliar treatments. While many natural enemies attack the stemborer and some are highly effective, they do not always protect the rice from losses (Khan et al. 1991).

While a *Bt* transgenic rice deployment system has much appeal for controlling the yellow stemborer, it would likely encounter problems with insect resistance especially in large homogenous plantings of transgenic rice. Rice appears to be the insect's sole host plant (Khan et al. 1991), therefore all members of populations residing in areas planted to *trnsgenic* rice could be exposed to the *Bt* toxins.

This paper reviews progress in identifying toxins suitable for use against the yellow stemborer and discusses potential strategies for prolonging the usefulness of *Bt* in engineered rice to control this pest. It is based on the approach that the International Rice Research Institute (IRRI) and collaborators are pursuing with the stemborer.

BT TOXINS FOR STEMBORER CONTROL

The first step in engineering any crop plant with an effective *Bt* deployment system is to find gene products that are highly active against the target pest. Measurement of specific activity of crystal proteins of *Bt* strain isolates is essential in pinpointing the genes. IRRI and Plant Genetic Systems of Gent, Belgium collaborated in isolating and characterizing strains of *Bt* for potential use against the yellow stemborer and other rice insect pests. The effort accumulated almost 4,000 *Bt* isolates from different Philippine environments, and about 1,000 strains have been selected from the isolates (Peferoen et al. 1991). The crystal proteins of specific *Bt* isolates from the Philippines and from other sources have been assayed.

Bioassays initially consisted of placing second instar yellow stemborer larvae on 14-day old rice seedlings that had been dipped in *Bt* suspensions of different concentrations. The technique

produced erratic results. The larvae apparently consumed insufficient amounts of the toxins before boring inside the plants where they were protected. Uneven distribution of the toxins on the dipped plants probably also contributed to the erratic results. As an alternative, we devised a bioassay using artificial diet supplied by Bio-Serv, Inc. (Frenchtown, N.J., U.S.A.) for rearing the beet armyworm, *Spodoptera exigua* (Hübner), and modified by A. T. Angeles and G. E. Damasco (unpublished) for rearing the yellow stemborer at IRRI. The diet is not adequate for complete yellow stemborer development, but the larvae live long enough to complete the bioassays.

Bt toxins are mixed into the liquid artificial diet (temperature below 60℃) and the diet-toxin mixture is poured into 20 ml scintillation vials without lids. The mixture is dried for 12 hr under a fumehood with the blower off or for 4 hr with blower on. For each bioassay, five concentrations of the toxin are tested with a minimum of 48 larvae/concentration plus two sets of untreated controls (minimum of 48 larvae/set). Before introducing stemborers into the vials (6, 1-day old larvae/vial), we slit the diet's surface (5 slits/vial) with a small spatula. The slits ease larval entry into the diet. We hold larvae in the vials at 27℃ for 4 days, record mortality, and use Probit analysis to compute the lethal concentration (LC) values.

Using the artificial diet bioassay, we have determined the toxicity of several purified CryI toxins and one CryII toxin. Table 1 shows results of one bioassay. In this bioassay, CryIA(b) was the most effective of the 8 toxins. The toxin was 5 times more toxic than the second most effective toxin, CryIA(c), at the LC_{50} level/but only about twices as toxic at the LC_{90} level/CryIIA was the third most effective toxin. These results indicate that genes of CryIA(b), CryIA(c), or CryIIa would be the first choices in efforts to engineer *Bt* genes in transgenic rice to control the yellow stemborer. However, other bioassays at IRRI using the same procedures showed CryIA(c) to be more effective than CryIA(b), although both were effective.

The artificial diet technique is an improvement over the plant dipping technique, but it also produced erratic results in some yellow stemborer bioassays. Mortality in the control group was unusually high (50% or more; 5-15% is acceptable) in those bioassays, for reasons that are not clear.

IRRI's experience stresses the importance of having an effective bioassay procedure and repeating it until the results consistent show that the *Bt* toxins are effective. If a wrong *Bt* gene is used, a genetic engineer could move the *Bt* gene codes into rice, achieve high levels of expression,

Table 1. Effect of *Bt* purified toxins on yellow stemborer larvae

Bt toxins	LC_{50} ± SE μg/ml	LC_{90} ± SE μg/ml	Slope ± SE
Cry IA(a)	19.68 ± 16.03	1024.24 ± 835.38	1.27 ± 0.39
Cry IA(b)	0.16 ± 0.07	2.87 ± 0.72	1.28 ± 0.32
Cry IA(c)	0.80 ± 0.36	5.63 ± 2.40	1.48 ± 0.18
Cry IB	11.55 ± 9.43	603.32 ± 491.77	0.75 ± 0.19
Cry IC	10.64 ± 9.19	589.79 ± 470.62	3.55 ± 2.38
Cry ID	18.05 ± 8.72	1.0E+08 ± 8.9E+04	0.55 ± 0.09
Cry IE	515.09 ±428.67	1.5E+09 ± 1.32E+09	1.56 ± 0.94
Cry IIA	1.41 ± 0.41	34.90 ± 2.57	3.92 ± 2.79

but produce a plant that fails to control the target pest. An inadequate bioassay, such as the plantdipping technique used initially, or the failure to repeat procedure could lead to an erroneous choice of genes.

USE OF MULTIPLE TOXINS

Van Rie et al. (1990) provided evidence that resistance in a strain of Indian meal moth, *Plodia interpunctella*(Hübner), was mediated by heritable change in toxinmembrane binding at receptor proteins in the insect's midgut. Resistance to CryIA(b) toxin was mediated by heritable changes in the toxin binding affinity of receptor proteins in epithelial cells of the insect's midugt. However, the resistant insects had a slightly greater than normal reduction in affinity of these receptors for the CryIA(b) toxin. The data would indicate that by using *Bt* toxins with different binding properties, in combination or sequentially, resistance could be prevented or delayed.

However, Gould et al. (1992) presented data that challenge this view, They showed that a strain of insects resistant to one *Bt* toxin may exhibit cross-resistance to other *Bt* toxins, even if the toxins differ significantly in chemical structure and activity. A laboratory strain of tobacco budworm, *Heliothis virescens* (F.), developed a 50-fold level of resistance after 17 generations of exposure to CryIA(c). The resistant strain exhibited resistance also to CryIA(a), CryIA(b), CryIB, and CryIC. Further, the resistance in this strain did not exhibit significant changes in toxin binding, and resistance was inherited as an additive trait when the budworm larvae received high doses of CryIA(c).

This recent finding of cross-resistance to *Bt* toxins defies the earlier held view about *Bt* resistance management. Previously, it appeared that resistance management was possible simply by replacing the no longer effective *Bt* toxin with another toxin. The study of Gould et al. (1992) clearly indicates that selection with a single *Bt* toxin could lead to broadly based resistance, therefore thwarting control of an insect population with any *Bt* product. The discovery that resistance was inherited as an additive trait has further implications. If resistance to the toxins is inherited as an additive or dominant trait, and if the toxins degrade over time or are used at low dosages, large populations can adapt to two toxins used in combination as fast or as faster than they would adapt to the two when used separately (Gould 1991).

Without data such as Gould et al. (1992) obtained for *H. virescens*, it presently is not possible to speculate on the potential for cross-resistance in the yellow stemborer. IRRI, North Carolina State University, University of Maryland, and Ohio State University presently are collaborating on studies of resistance and cross-resistance to *Bt* toxins. Simultaneously, IRRI is gearing up to engineer rice with *Bt* genes for evaluation in containment facilities, Plans are to develop and compare the following *Bt* deployment systems in transgenic rice: CryIA(b) or CryIA(c) alone; CryIIA alone; and CryIA(b) or CryIA(c) and CryIIA together. CryIIA(b), CryIA(c), and CryIIA are the most effective toxins tested against the yellow stemborer so far. The two CryI proteins are structurally similar based on amino acid sequence and their corresponding activities, but each differs significantly from CryIIA(Höfte and Whiteley 1989). By comparing the mono- and bi-

toxin deployment systems, we will determine the added value, if any, of combining toxins quite different in structure to slow the rate of adaptation of stemborers to *Bt*.

In addition, IRRI will be examining more of its *Bt* strain collection for new toxin sources. There is a possibility that even more effective toxins will be discovered.

MINIMIZING EXPOSURE

Evolutionary models predict that the lower the overall selective pressure challenging a pest is the longer it will take for the pest to adapt to a toxin(Gould 1988b). The idea of minimizing pest exposure to a control tactic in fact is a basic precept of integrated pest management (Smith & van den Bosch 1967), which rejects the notion that the presence of a pest species necessarily justifies action for control. Low-level infestations of insect pests generally are not harmful and may be desirable, providing important sources of food or reproductive hosts for natural enemies.

Tissue- and Time-Specific Expression

As one way to minimize the exposure to target insects, Gould (1988a) proposed using promoters that express *Bt* toxins only in specific plant tissues or at specific times. Expression in the most pest-sensitive plant parts or at certain times might provide adequate protection while affecting only a portion of the pest population.

Recent information on the feeding behavior of yellow stemborer on rice plants strengthen the argument for a tissue- and time-specific promoter system (V.F. Magalit & D.G. Bottrell, unpublished). Most young stemborer larvae selectively seek the young tillers' first and second leafsheaths and growing shoots, whereas older larvae feed inside the stems (internodes) (Fig. 1). This feeding pattern is similar for larvae on rice in vegetative, panicle initiation, and booting stages. The observations on feeding behavior were in the laboratory using pot-grown plants. Observations on the field behavior of yellow stemborer larvae are necessary to confirm this feeding pattern.

Injury made by larvae cutting the young leafsheaths of plants in the vegetative stage may prevent the affected tillers from bearing panicles (a condition known as "deadheats"). However, a high-tillering variety can produce replacement tillers so a vigorous, well-nourished crop generally does not suffer yield loss when attacked in the vegetative stage. Larval injury during panicle initiation and booting stage is potentially much more serious, resulting in unfilled grains ("Whiteheads") at harvest time (Khan et al. 1991).

It therefore appears that the *Bt* toxins might be expressed only after plants reach the panicle initiation stage and then confined to the first and second leafsheaths and growing shoots. *By* delaying expression until panicle initiation, rather than expressing the toxins each crop season (Table 2). The tissue-specific expression system would reduce exposure further since about 30 % of the young larvae do not feed on the first and second leafsheaths and growing shoots from panicle initiation onwards (V.F. Magalit and D.G. Bottrell, unpublished).

The University of Ottawa is collaborating with IRRI to develop a spatial and temporal *Bt* toxin

Fig. 1. Distribution of yellow stemborer larvae(blackened areas of plants) at different ages (1-21 days) on rice in panicle initiation to booting stage. The rice was infested with neonate larvae when in the panicle initiation stage.

Table 2. Association of yellow stemborers on rice in prepanicle initiation and later stages

	Pre-panicle	Panicle initiation and later
Potential for stemborer losses	low	moderate to high
No. stemborer generations	1	1
Estimated percentage of young stemborer larvar feeding on first and second leafsheaths and growing shoots	80	70

expression system in rice. When the transgenic rice plants with the selective promoters are available, we will test them experimentally for efficacy against the stemborer under contained conditions. Also, we will conduct selection experiments over a series of consecutive generations to compare the rate of stemborer adaptation under a selective and complete system of expression.

Exogenous Chemical Control

Another way of achieving time-specific *Bt* expression might be through use of some external stimulus. Gould(1988b) discussed the possibility of using a gene expression system to produce toxins only when a predetermined amount of pest damage has occured. Williams et al. (1992) devised an external triggering system for temporarily controlling the expression of *Bt* toxins in tobacco. They constructed a gene composed of the delta-endotoxin coding sequence from *Bt* fused to the promoter of the pathogenesis-related protein Ia(PR-Ia) gene from tobacco. Expression of the native PR-Ia gene is inducible by inoculation with a variety of pathogenes, as well as several chemical stimuli including salicylic acid and polyacrylic acid.

The chemical regulation of *Bt* toxin expression in trans genic plants may have potential in areas where farmers have resources to monitor fields regularly and apply the chemical regulator treatments properly. The system seems to be impractical in areas where the farmers have limited resources.

Mixing Transgenic and Non-Transgenic Plants

Another potential way to minimize exposure to the stemborer is by mixing transgenic rice containing *Bt* genes with non-transgenic rice. Atsatt and O'Dowd (1976) argued that the presence of susceptible host species or varieties could lengthen the useful life of resistant hosts by slowing pest evolution. Simulation models by Gould (1986) for mixing resistant and susceptible varieties of wheat to retard adaption of the Hessian fly, *Mayetiola destructor* (Say), support this hypothesis. For more than two decades, plant pathologist have advocated use of mixtures of plant genotypes or varieties for disease control for similar reasons (e.g., Browning & Frey 1969).

Mixtures could be achieved by planting random mixtures of transgenic and non-transgenic rice. The non-trnsgenic rice plants would serve as escape refuge for the stemborers similarly as the untreated plant parts would serve this purpose in a tissue-or time-specific *Bt* expression system. The merit of using planting mixtures needs to be determined experimentally, comparing

stemborer control and rate of adaptation over time using different transgenic/non-transgenic planting ratios.

An alternative to planting random mixtures of transgenic and non-transgenic rice would be to rotate transgenic and non-transgenic plantings or plant the transgenic rice only in areas prone to heavy stemborer infestations.

CONCLUTION

If current biotechnology research programs ignore the mounting evidence about the potential problem of resistance to *Bt*, they increase the risk of losing a potentially useful pest control tool. If not bridled carefully, *Bt* in engineered plants should not ensure any more defense against insect pest adaption than the *Bt* sprays, which already have lost their effectiveness against the diamondback moth in some areas (Tabashnik et al. 1990, Shelton & Wyman 1991). As Gould (1991) concluded, pests are masters at surviving the farmer's assaults and any new control strategy must anticipate pests' evolutionary responses.

Current biotechnology research is focused heavily on engineering crops with single *Bt* genes expressed in all tissues and at all times in the growing season. This approach is somewhat reminiscent of developments after World War II when new synthetic insecticides emerged. Enthusiasm for the new materials tended to palliate the potential problem of resistance. Yet, most entomologists today would cite the problem of genetic resistance in pests as the chief factor dimming prospects for continued use of insecticides. Ironically, the problem of resistance to chemical insecticides has been a major driving force behind the exploitation of *Bt*.

Bt does have many features desperately needed in rice protection. However, its exploitation must not stop at the molecular level. Prolonging its usefullness will require the cooperation of teams of biotechnologists, popultation geneticists, ecologists, and pest management specialists. The goal should not be to see how little time is required to develop a *Bt* transgenic system. Rather, it should be to see how long they can prolong ito usefulness against the pests.

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