# An alternative strategy for sustainable pest resistance in genetically enhanced crops

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***Bacillus thuringiensis*** **(Bt) crystal protein genes encode insecticidal δ-endotoxins that are widely used for the development of insect-resistant crops. In this article, we describe an alternative transgenic strategy that has the potential to generate broader and more sustainable levels of resistance against insect pests. Our strategy involves engineering plants with a fusion protein combining the δ-endotoxin Cry1Ac with the galactose-binding domain of the nontoxic ricin B-chain (RB). This fusion, designated BtRB, provides the toxin with additional, binding domains, thus increasing the potential number of interactions at the molecular level in target insects. Transgenic rice and maize plants engineered to express the fusion protein were significantly more toxic in insect bioassays than those containing the Bt gene alone. They were also resistant to a wider range of insects, including important pests that are not normally susceptible to Bt toxins. The potential impact of fusion genes such as BtRB in terms of crop improvement, resistance sustainability, and biosafety is discussed.**

Bt genes | transgenic plants | transgenic maize | transgenic rice

Transgenic plants expressing *Bacillus thuringiensis* (Bt) toxins have been used successfully to provide resistance against selected insect pests for several years. Indeed, insect resistance is the second most widely used trait in transgenic crops (after herbicide tolerance) in world agriculture (1–5). One potential problem with Bt genes is that Bt insecticides are very widely used, with up to 90% of microbiological insect control products based on topically applied Bt toxins. For this reason, there is concern that insects might evolve resistance to Bt toxins (6, 7). The diamondback moth (*Plutella xylostella*) has evolved resistance in some open field populations in response to repeated exposure to foliar sprays containing Bt proteins (8), whereas laboratory selection experiments with other insect pests have shown that recessive mutant alleles can confer resistance to multiple Bt toxins (7–10). However, note that the evolution of resistance in insects against transgenic plants expressing Bt toxins has yet to be seen in the field.

Recent strategies to address potential limitations in conventional transgenic insect pest control include the stacking or pyramiding of multiple transgenes in the same transgenic plant (11) and the use of hybrid toxins (e.g., fusions between a synthetic truncated Cry1Ba and domain II from Cry1Ia; ref. 12). We have devised an alternative strategy in which the Bt toxin Cry1Ac is fused to the nontoxic ricin B-chain (RB). The recognition of toxin-binding sites in the insect midgut is an important factor determining the spectrum of Bt toxin activity and the severity of toxemia (13). Several groups investigating the mechanism of toxin recognition have identified *N*-acetyl galactosamine residues as an important component of Bt toxin-binding receptors (14–16). Therefore, we selected the ricin B subunit as a fusion partner for the Bt toxin because RB is a galactose- and *N*-acetylgalactosamine-specific lectin that binds such residues with very high affinity (17). Recent reports support a broader role for RB in the delivery of the highly toxic ricin A chain. The RB polypeptide is thought not only to interact with cell surface galactose residues, but it also may be involved in the intracellular trafficking of the ricin toxin (18). We envisaged that a fusion protein comprising an N-terminal Bt toxin and a C-terminal RB polypeptide (which we here designate BtRB) would provide a unique-binding domain that would allow the hybrid protein to bind to a wider repertoire of receptors than the control toxin (in this case, Cry1Ac). Furthermore, the fact that single alleles in homozygous form can confer resistance to Bt toxins suggests that each toxin interacts with a single receptor, and loss or modification of this receptor leads to resistance in otherwise susceptible insects. Therefore, by increasing the number of binding domains on each toxin, the likelihood of resistance evolving in target populations is reduced because mutations affecting several different receptors are highly unlikely to occur simultaneously.

The results of insect bioassays showed that transgenic rice and maize plants expressing the Cry1Ac-RB fusion protein were significantly more toxic to a range of insect pests than those expressing Cry1Ac alone. Furthermore, the fusion protein conferred resistance to a broader spectrum of insect pests than those normally susceptible to Cry1Ac.

Wider application of this technology will necessitate the testing of a broad range of insects encompassing agriculturally important pests and also beneficial insects to ascertain the full efficacy and safety of these fusion proteins. This article sets the stage for developing an alternative strategy by using transgenic plants for the control of important insect pests in a sustainable manner.

## Materials and Methods

**Site-Directed Mutagenesis of the RB.** Four mismatched oligonucleotides were used to introduce unique EcoRI and HindIII restriction sites at selected positions in plasmid pBWT [containing the nontoxic RB gene (19)]. PCR mutagenesis was carried out in a 50-μl total volume containing 1× PCR buffer (Roche Molecular Biochemicals), 200 μM each dNTP, 15 mM $MgCl_2$, 300 nM each primer, 2.6 units of enzyme mix (Roche Molecular Biochemicals), and 70 ng of pBWT plasmid DNA. After an initial denaturation step at 94°C for 2 min, 10 fixed amplification cycles were carried out (94°C for 15 s; 65°C for 30 s; and 72°C for 1 min), followed by 20 progressively lengthening cycles (94°C for 15 s; 65°C for 30 s; and 72°C for 1 min; elongation increasing by 5 s each cycle). A final extension step was carried out at 72°C for 7 min. PCR products were sized by 0.8% agarose gel electro-

Abbreviations: Bt, *Bacillus thuringiensis*; RB, ricin B-chain.

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phoresis, purified (QIAquick gel extraction kit, Qiagen, Valencia, CA), and subcloned in the vector pGEM-T (Promega). Insert size and orientation was confirmed by digestion with EcoRI and HindIII. Sequencing was carried out by using M13/pUC19 primers (GIBCO/BRL) and the Big Dye sequencing kit (Roche Molecular Biochemicals). Cycle sequencing was carried out by using the PTC-200 Peltier thermal cycler (MJ Research, Cambridge, MA). Primer sequences were as follows, with mutated residues shown in italics: LF1, 5′-CAACAACAAAG-*GAA*TTCATGCTGATG-3′; LF2, 5′-CCATGCAAGTC-*G*AATTCAGATGCAAATCAG-3′); LB1, 5′-GGACACACA-CACTGCAAG*CTT*GTAATC-3′; LB2, 5′-CGGATCCGA-*AA*GCTTCACATCTAACAC-3′; and LB3, 5′-GCTTG-CAAGC*TT*AGACCATATAGCCC-3′.

**Expression Constructs and Plant Transformation.** The *cry1Ac* gene was excised from its source plasmid, pUBC (20), by digestion with BamHI and EcoRI and was subcloned into an intermediate vector. This recombinant plasmid was digested with EcoRI and HindIII, allowing directional subcloning of the RB gene fragment described above. Thus, a second intermediate construct was generated in which the Bt gene was joined to the RB fragment. This construct was digested with EcoRI and XhoI, and the termini were polished by using mung bean nuclease, thus bringing the Bt- and RB-coding regions in-frame upon religation. The recombinant plasmid was then digested with StuI and HindIII, releasing the fusion cassette for directional subcloning back into the original vector. Parallel digestions with BamHI and EcoRI, and with EcoRI and HindIII, allowed subcloning of the unmodified *cry1Ac* and RB genes, respectively, as controls. The control and fusion protein cassettes were isolated from the intermediate vectors by using EheI and HindIII and directionally subcloned in pAL76 (a ubiquitin promoter-based transformation vector) previously digested with SmaI and HindIII. Transformation of maize and rice embryogenic callus was carried out by particle bombardment as described (21, 22). In each transformation procedure, the maize callus was cobombarded with a plasmid carrying the *bar* selectable marker for phosphinothricin resistance, and the rice callus was cobombarded with the *hpt* selectable marker for hygromycin resistance.

**Southern Blot Analysis.** Genomic DNA was isolated from the fresh leaf tissue of transgenic and control plants by using the Phytopure plant DNA-extraction kit (Amersham Pharmacia). Aliquots of DNA (20 $\mu$g) were digested with 50 units of HindIII overnight at 37°C. The DNA (10 $\mu$g/lane) was fractionated by 0.8% agarose gel electrophoresis, denatured, neutralized, and blotted onto Hybond-$N^+$ nylon membranes (Amersham Pharmacia). The probes for *cry1Ac* and RB were synthesized by PCR by using the intermediate vectors as templates (see above). Generic primers were used to amplify the inserts in the presence of $\alpha$-[$^{32}$P]dCTP. Southern blots were prepared and hybridized according to standard procedures (23).

**Western Blot Analysis.** Western blot analysis of transgenic plants was carried out on small leaf sections ground to a fine powder under liquid nitrogen. Samples were dispersed in protein-extraction buffer (100 mM Tris·Cl, pH 8.1/100 mM 2-mercaptoethanol) and centrifuged at 12,000 $\times$ *g* for 10 min at 4°C. The samples were fractionated by SDS/PAGE and transferred to nitrocellulose membranes (Hybond C, Amersham Pharmacia) by using the TransBlot semidry transfer cell (Bio-Rad) according to the manufacturer's instructions. Filters were probed with antisera against Cry1Ab (which crossreacts strongly with Cry1Ac). Bound Ab was detected by using horseradish peroxidase-conjugated anti-rabbit IgG as the secondary Ab (BioRad), followed by visualization by using the enhanced chemiluminescence method (Amersham Pharmacia) according to the supplier's protocols.

**Insect Bioassays.** Insect cultures were held and reared under a Department for Environment Food and Rural Affairs (U.K.) license. A culture of striped stem borer eggs (*Chilo suppressalis*) was obtained from M. Cohen (International Rice Research Institute, Laguna, Philippines). The eggs were maintained under a 27/25°C day/night temperature regime with a 16-h photoperiod. Bioassays were carried out on 7-cm stem sections from transgenic maize plants, rice plants, and age-matched WT controls. Each stem section was placed on moist filter paper in a Petri dish and infested with six (rice) or 10 (maize) neonate stem borer larvae (<15 h old). Four replicates were set up for each transgenic rice line, and eight replicates were set up for the WT controls, whereas seven replicates were set up for the maize lines. The dishes were then sealed with Parafilm and left for 5 days in a controlled environment growth chamber. After the trial period, stem sections were dissected under a binocular microscope and insect survival, development, and weight were recorded. Cotton leaf worm (*Spodoptera littoralis*) eggs were maintained at 25 $\pm$ 2°C with a 16-h photoperiod and 70–80% relative humidity. Bioassays were carried out as described above, by using cut leaf segments. Each segment was infested with 10 neonate *S. littoralis* larvae (<20 h old) by placing them on the upper surface of the leaf section. Survival was monitored over 9 days and recorded on days 6 and 9. The experiment was carried out three times under the same conditions. Aphids (*Rhopalosiphum padi*) were maintained and tested under the same conditions as used for *S. littoralis*, except that mesh-covered boxes rather than Petri dishes were used to contain the insects. Five neonate *R. padi* larvae were placed in each box, and the leaf sections were refreshed every 4 days. Leafhoppers (*Cicadulina mbila*) were tested in Petri dishes containing a small amount of 1% agar and with mesh-covered ventilation slits in the lids. Leaf discs (10 cm) were placed in these chambers and infested with hopper nymphs. Bioassays were carried out over 5 days at 25°C, and survival was recorded on days 2, 4, and 5. For each plant line, 10 replicates, each of five nymphs, were carried out. The experiment was carried out three times under the same conditions.

**Statistical Analysis.** Statistical analysis of insect data were performed with STATVIEW software (version 5.0; Abacus Concepts, Berkeley, CA). ANOVA was used to test for significant differences between treatments, with a rejection limit of $P > 0.05$.

## Results and Discussion

**Design of the BtRB Fusion Protein.** A construct was assembled that encoded domains I–III of the Bt Cry1Ac toxin, equivalent to the active toxin produced by proteolysis of the protoxin, fused N-terminally to the complete sequence of the nontoxic carbohydrate-binding B-chain of ricin via a 4-aa linker region. Therefore, the complete polypeptide was 881 aa in length with a predicted $M_r$ of 98,174.

**Transformation and Regeneration of Transgenic Plants.** Embryogenic callus from mature maize and rice seeds was bombarded with the BtRB fusion construct in combination with the appropriate selectable marker, resulting in the recovery of transgenic maize and rice lines carrying the fusion gene. Additional transformations were carried out to generate control transgenic maize and rice plants carrying the *cry1Ac* gene or the RB gene fragment. Representative Southern blots of genomic DNA from transformed maize plants are shown in Fig. 1*A*. These data confirm that the transgenes integrated into the maize genome and that three to 10 copies of the transgene per haploid genome are present in the lines tested. Similar data were generated for transgenic rice plants (data not shown).

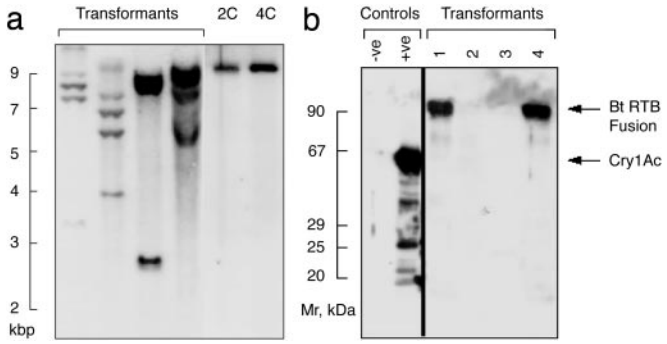


**Fig. 1.** DNA and expression analysis. (*a*) Southern blot analysis of genomic DNA (10 $\mu$g per lane) from independent primary maize transformants, digested with HindIII, which cuts the transforming plasmid once. The DNA was hybridized with a probe specific for BtRB; controls gave no detectable hybridization. The designations 2C and 4C are plasmid copy number controls. (*b*) Immunoassay of transgenic maize plants expressing BtRB fusion protein (lanes 1 and 4). Nontransformed and transgenic *cry1Ac* plants were used as negative and positive controls, respectively. Lanes 2 and 3 are negative segregants. Protein extracted from leaf samples (100 $\mu$g of total protein per lane) were separated by SDS/PAGE (5% acrylamide) and electroblotted onto nitrocellulose. Bt Cry1Ac and the BtRB fusion protein were visualized by enhanced chemiluminescence using anti-Cry1Ab and horseradish peroxidase as primary and secondary Abs, respectively.

**Expression of the Fusion Protein in Transgenic Plants.** Expression of the BtRB fusion protein in transgenic rice and maize plants was demonstrated by immunoassay by using anti-Cry1Ab Abs, which crossreact strongly with Cry1Ac (Fig. 1*B*). Western blot analysis confirmed the presence of a single polypeptide of the correct predicted molecular weight. In the transgenic maize plants, a major band of ≈98 kDa was clearly visible, with a second very faint band of ≈70 kDa (Fig. 1*B*). No band corresponding to the expected size of Cry1Ac (≈60 kDa) could be detected in the fusion plants, but a band of this size was seen in the Cry1Ac control plants (Fig. 1*B*, +ve control). Analysis of subsequent generations of fusion plants confirmed that the transgene was stably inherited. Similar results were obtained for rice.

**Insect Bioassays.** To test our hypothesis that increasing the binding capacity of Bt δ-endotoxins by fusion with the carbohydrate-binding domain from the RB would increase the effective range of susceptible insects, bioassays were carried out against a range of insect species, including examples known to be susceptible to Cry1Ac, or tolerant to this insecticidal protein. For all insect bioassays, we selected transgenic rice and maize plants expressing the fusion and control toxins at similar levels. We deliberately chose transgenic plants that expressed Cry1Ac and the BtRB fusion at low levels to allow a clear discrimination between the two populations.

The stem borer (*C. suppressalis*) is normally susceptible to Cry1Ac. In maize plants expressing Cry1Ac (Fig. 2*A*), survival of stem borer larvae was reduced by ≈17% compared with either nontransformed control plants or the negative segregants. However, in maize plants expressing the fusion protein, survival of stem borer larvae was further reduced by ≈75%, compared with control plants. As expected, expression of the control RB alone had little effect on survival. Expression of the BtRB fusion protein in rice produced similar results (Fig. 2*B*). In this case, expression of Cry1Ac reduced survival by 20–30%, whereas expression of the BtRB fusion reduced survival by 60–90% in plants derived from three independent transformations. The differences between plants expressing the fusion proteins and other treatments are significant for both host species. With both maize and rice, expression of the fusion protein dramatically reduced insect growth, with corresponding reductions in the level of tissue damage (Fig. 2). Expression of the unmodified Bt toxins did not result in high insect mortality in these assays. Higher mortality levels have previously been reported in rice plants expressing Cry1Ab, but the authors also reported high levels of unrecovered larvae and elevated mortality on control plants (24, 25). In contrast, in this study, survival on control plants was high (>95%). The present results clearly demonstrate that although expression of Cry1Ac in both cereals at relatively low levels did have a deleterious effect on survival of stem borer (statistically significant at $P < 0.05$ for assays in rice), its efficacy was significantly enhanced by expression as a fusion protein with the ricin B chain in the form of BtRB.

In contrast to stem borer, cotton leaf worm (*S. littoralis*) is

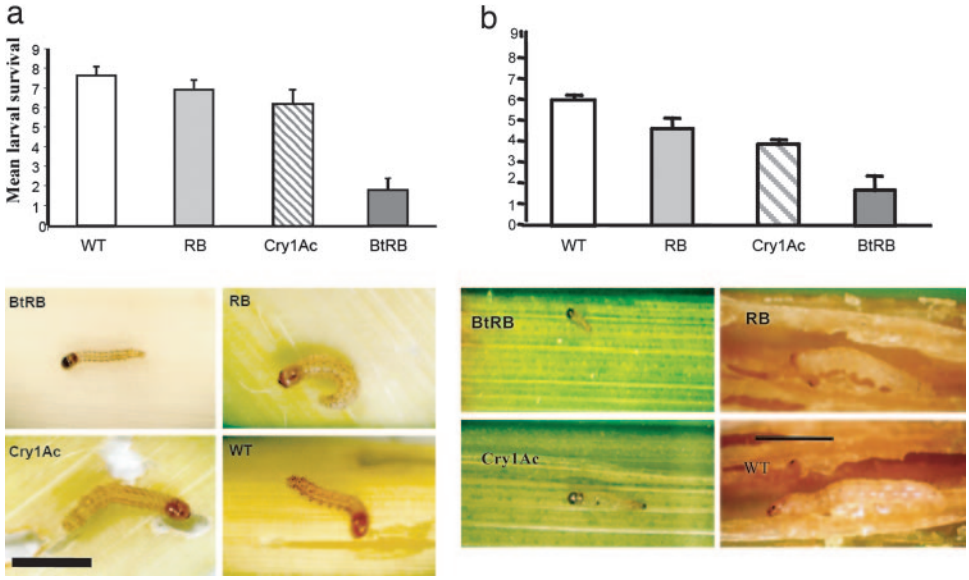


**Fig. 2.** Insect bioassays. Effects of BtRB fusion protein on mean survival and development of stem borer (*C. suppressalis*) in transgenic maize (*a*) and rice (*b*) plants. WT (WT nontransformed plants) were used as negative controls; plants expressing Cry1Ac toxin and RB were used to determine the enhancement in toxicity provided by the fusion protein. BtRB, Cry1Ac/RB fusion protein. Differences between BtRB and other treatments in both assays are significant at $P < 0.05$; other differences are not significant. (Scale bar, 2 mm.)

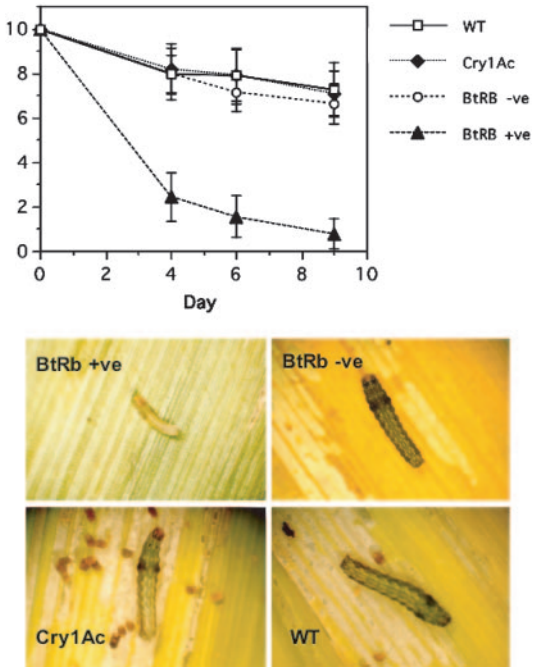


**Fig. 3.** Effects of BtRB fusion protein on mean survival and development of cotton leaf worm (*S. littoralis*) on R1 transgenic maize plants. WT (WT, nontransformed plants); BtRB −ve (negative segregants); BtRB +ve (positive segregants). Differences between BtRB +ve and other treatments are significant at $P < 0.001$ from day 4. Vertical axis represents mean larval survival.

tolerant to Bt δ-endotoxins and so provides a good model on which to test our hypothesis. Segregating R1 maize plants expressing the fusion protein were infested with neonate larvae and effects on survival and development were monitored throughout the course of the trial. By day 4, almost 78% of the larvae had died on plants expressing the fusion protein, increasing to >90% by day 9. In contrast, mortality on control plants (nontransformed and negative segregants) or those expressing Cry1Ac alone was <20% by day 4, with levels remaining similar throughout the duration of the trial (Fig. 3). Furthermore, this reduction in survival correlated with the rate of insect development. After 9 days, any surviving larvae present on the plants expressing the fusion protein had only reached the second instar, whereas those on the other plants had developed to the third instar by 7 days (Fig. 3). This result confirms the insensitivity of *S. littoralis* to Cry1Ac, but it shows that fusion of the toxin to the ricin domain has extended the range of toxicity of Cry1Ac to include this species.

Bioassays were also carried out with the leafhopper *C. mbila*, a homopteran plant pest, because Bt toxins with activity toward species in the order Homoptera have not been reported. The present study confirmed the lack of toxicity of Cry1Ac toward these sap-sucking insects (Fig. 4). Survival was ≈80% over 4 days on WT plants, and there were no significant differences in survival on plants expressing the toxin. However, survival on R1 maize plants expressing the BtRB fusion protein was reduced to ≈5% after 4 days, whereas segregating nonexpressing plants gave results similar to the WT controls. These results show that Bt toxins with additional binding domains can generate unique insecticidal activity. However, the fusion protein is not toxic to all homopteran insect species because a bioassay of the cereal aphid *R. padi* on transgenic maize plants showed no evidence for the toxicity of either Cry1Ac or the BtRB fusion protein (data not shown).

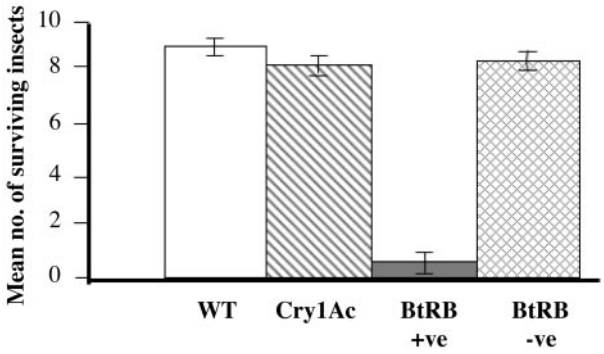


**Fig. 4.** Effects of BtRB fusion protein on mean survival of leafhopper (*C. mbila*) on R1 transgenic maize plants. Differences between BtRB and other treatments are significant at $P < 0.001$. (Scale bar, 2 mm.)

**Mechanism of BtRB Specificity.** If transgenic crops expressing fusion proteins like BtRB were adopted by farmers, it would be necessary to establish that the insecticidal activity of the unique toxin retained some specificity, to avoid deleterious effects on nontarget and beneficial insect species. As stated above, the BtRB fusion demonstrated enhanced toxicity toward the stem borer, which is known to be susceptible to Cry1Ac, and toward cotton leaf worm, which is tolerant. The fusion also showed toxicity toward the leafhopper, a homopteran pest normally unaffected by any of the Bt toxins. However, the fusion protein was not indiscriminate in its range of activity because it had no significant effect on cereal aphids. The basis of the specificity of insecticidal activity shown by the BtRB fusion protein remains to be established, although the data presented suggest that the additional binding domain is responsible for the wider range of its effectiveness. Proteins extracted from lepidopteran gut were probed with ricin after separation by SDS/PAGE, and ricin binding was detected by anti-ricin Abs. These blots showed that the lectin was capable of binding to at least 10 polypeptides in the $M_r$ range of 20–100 kDa. Cry1Ac itself is thought to bind to a specific receptor in the lepidopteran gut, aminopeptidase N (26, 27), although it also binds to other molecules such as cadherin (28) and a range of other gut proteins (29). Binding to aminopeptidase N is specifically inhibited by *N*-acetylgalactosamine, and the toxin has a binding pocket for this sugar (30). Thus, the broader specificity of the RB, which binds to both galactose and *N*-acetylgalactosamine, should lead to an increased range of putative receptors for the fusion protein. However, the toxicity of Cry1Ac is not solely dependent on *N*-acetylgalatosamine-mediated binding (31), and the increased toxicity of the fusion protein may also be due to improved membrane insertion mediated by the RB.

**Potential Benefits and Drawbacks of BtRB Crops.** Standard Bt cotton, maize, and potato crops have been released commercially and have been readily adopted by farmers (32), whereas Bt rice, although yet to be commercialized, has been tested successfully in several field trials (33). Experience has shown the benefits of such enhanced crops in terms of increased yields, reduced chemical inputs and, as a knock-on effect, improved farmer and consumer health. Sustainable resistance against insect pests is the cornerstone of any sensible deployment strategy that uses transgenic plants expressing insecticidal proteins, either alone or, preferably, within an integrated pest management system (32, 34). It is in the context of resistance management that crops expressing fusion proteins such as BtRB could be the most beneficial. To our knowledge, there have been no reports of natural insect populations evolving resistance to Bt crops in the wild, in part reflecting the fitness cost of resistance alleles, but

mainly because of the implementation of resistance strategies (35). Bt crops in the United States must be comaintained with refuges that decrease the selection pressure on target pests and reduce the likelihood of resistance becoming established, whereas in China, refuges in Bt cotton crops are provided by an alternative host plant that supports the major pest species, *Helios armigera* (36). This type of mandatory refuge management system may be difficult to implement for crops such as rice and maize in developing countries with many smallholder farms and where substitute hosts for insect pests are not available. As an alternative measure, it has been suggested that crops should be developed expressing multiple toxins at high doses, so that insect populations would have to undergo the highly unlikely process of acquiring several simultaneous mutations to become resistant (11, 37, 38). Fusion proteins such as Cry1Ba/Cry1Ia (12) and BtRB should provide strong and sustainable resistance requiring multiple counteradaptive mutations but would require only a single toxin transgene.

Lessons learned after the indiscriminate and irresponsible use of chemical pesticides for the control of insect pests over the past several decades call for reason and caution in how we deploy transgenic plants expressing insecticidal genes in the present and in the future. However, this caution does not translate to a de facto moratorium on research to achieve such sustainability. On the contrary, bold and daring strategies need to be explored to test hypotheses and arrive at strategies that provide an overall balance of cost vs. benefit. Bt transgenic plants are remarkably specific in their activity, with little or no effect on nontarget organisms (32). Field tests with Bt rice, maize, and other crops have revealed no negative impact on biodiversity and indeed a positive impact resulting from the reduction in pesticide use (39, 40). It will be necessary to test fusion proteins, which alter the activity of the parent Bt toxins, rigorously in laboratories and field trials to determine which species are affected. Another strong advantage of conventional Bt crops is that there is no credible evidence for toxicity or allergenicity in humans (39). Again, it will be necessary to test new fusion proteins such as BtRB rigorously to make sure the same benefits are maintained. Although further work will be required to characterize the insecticidal activity and other properties of BtRB and similar fusion proteins, the results reported here provide the basis for a conceptual framework, which could lead to the development of unique and sustainable strategies for insect resistance.

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