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Can pyramids and seed mixtures delay resistance to Bt crops?

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Abstract

The primary strategy for delaying evolution of pest resistance to transgenic crops that produce insecticidal proteins from *Bacillus thuringiensis* (Bt) entails refuges of plants that do not produce Bt toxins and thus allow survival of susceptible pests. Recent advances include using refuges together with Bt crop "pyramids" that make two or more Bt toxins effective against the same pest, and planting seed mixtures yielding random distributions of pyramided Bt and non-Bt corn plants within fields. We conclude that conditions often deviate from those favoring success of pyramids and seed mixtures, particularly against pests with low inherent susceptibility to Bt toxins. For these problematic pests, promising approaches include using larger refuges and integrating Bt crops with other pest management tactics.

Key words: Cross-resistance, genetically engineered, redundant killing, resistance management, sustainability, transgenic.

Evolution of pest resistance threatens the benefits of transgenic Bt crops

The world's population is expected to grow from 7.2 billion now to at least 9.6 billion by 2100, greatly increasing demand for agricultural output [1-3]. Crops genetically engineered to produce insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) can help meet this demand by suppressing pest populations [4-7], increasing or stabilizing yield [8-10], reducing reliance on conventional insecticides [10-12], and enhancing favorable effects of beneficial arthropods [13-16]. From 1996 to 2014, farmers planted Bt crops on a cumulative total of 648 million ha worldwide, consisting almost entirely of Bt corn and Bt cotton [17]. Bt soy was planted in Brazil on a cumulative total of 7.4 million ha in 2013 and 2014 and Bt eggplant was planted commercially in 2014 on a small scale in Bangladesh [17]. Yield gains and insecticide reductions with Bt crops are often sufficient to increase farmer profits, which is the primary reason farmers use these crops in the United States [10, 12]. In the United States in 2015, Bt corn accounted for 81% of all corn and Bt cotton for 84% of all cotton [18].

As Bt crops have become more widely adopted some of their economic and environmental benefits have been lost because of rapid evolution of resistance by pests, particularly to the earliest commercialized Bt crops that produced only one Bt toxin [19] (Box 1 and Table 1). Since Bt crops were first commercialized 20 years ago, the refuge strategy has been the primary approach used to delay pest resistance [19, 20]. In this strategy, refuges of non-Bt host plants allow survival of susceptible pests that can mate with resistant pests emerging from Bt plants (Figure 1). Laboratory and greenhouse experiments, large-scale studies, and retrospective comparisons of patterns of field-evolved resistance show that refuges can delay resistance [19, 21-23]. This review focuses on two recent developments in managing resistance to Bt crops, both of which are refinements of the refuge strategy: using refuges in conjunction with Bt crop pyramids that have two or more toxins effective against the same pest, and planting random mixtures of Bt and non-Bt seeds.

Bt crop pyramids

Each of the original Bt crops commercialized in 1996 was engineered to make a single crystalline (Cry) toxin to kill larvae of some key lepidopteran pests [24]. To delay resistance, improve efficacy against some pests, and broaden the spectrum of pests controlled, most newer Bt crops produce two or more Bt toxins [20]. Current multi-toxin crops produce two or more Bt

toxins which belong to either the Cry protein family or the vegetative insecticidal protein (Vip) family (Table 2). Pyramided Bt crops are a special kind of multi-toxin crop designed to delay evolution of resistance by producing two or more distinct toxins that kill the same pest [20, 25]. First commercialized in 2003, such pyramids have become increasingly prevalent in recent years in the United States and other countries [19, 26]. For example in 2014, a pyramid producing Bt toxins Cry1Ac and Cry2Ab accounted for 96% of the 12 million ha of Bt cotton in India [27].

Conditions promoting durability of Bt crop pyramids

Five conditions that promote the durability of both single-toxin and pyramided crops are: 1) refuges are sufficiently abundant, 2) alleles conferring resistance are rare, 3) resistance is recessive, 4) fitness costs are associated with resistance, and 5) resistance is incomplete [19, 20]. Retrospective analyses show that all cases of field-evolved practical resistance to single-toxin crops involve substantial deviations from one or more of the first three conditions [19, 28, 29]. Conversely, previous reviews have concluded that fitness costs associated with resistance and incomplete resistance can increase the durability of Bt crops [30-32]. Here we synthesize theory and evidence about three conditions that are especially important for the durability of Bt crop pyramids: 6) each toxin in the pyramid can kill all or nearly all susceptible insects, 7) no cross-resistance occurs between toxins in the pyramid, and 8) pyramids are not grown concurrently with single-toxin plants that produce one of the toxins in the pyramid [19-21, 26].

Conditions 6 and 7 favor redundant killing, which occurs when an insect resistant to one toxin produced by a pyramid is killed by another toxin produced by the pyramid [26]. If the concentration of each toxin in a pyramid is high enough to kill all susceptible insects and no cross-resistance occurs between toxins, complete redundant killing occurs because only individuals with alleles conferring resistance to all toxins in the pyramid will survive on the pyramid. Moreover, if resistance to each toxin is rare (condition 2) and recessive (condition 3), only the extremely rare individuals homozygous for resistance to each toxin in the pyramid will survive on the pyramid (Figure 1).

The extent of redundant killing can been quantified using the redundant killing factor: (RKF) = 1 – [(proportion survival on pyramid for insects homozygous resistant to one toxin) – (proportion survival on pyramid for insects homozygous susceptible to both toxins)] [26]. RKF varies from 0 (no redundant killing) to 1 (complete redundant killing), with values markedly

lower than 1 projected to substantially accelerate the evolution of resistance [26]. In an analysis based on survival of three pests on different types of pyramids (n = 12 cases), RKF ranged between 0.81 and 1 [20].

Pyramids kill all or nearly all susceptible insects

Results from a mathematical model indicate that the concentration of each toxin of a two-toxin pyramid must be high enough kill at least 95% of susceptible individuals for pyramids to be most effective [25]. Assuming that each toxin acts independently, two-toxin pyramids are thus expected to be most effective when they kill at least 99.75% of susceptible insects [20]. In an analysis of nine pest-pyramid combinations, mortality on pyramids met this criterion in only half of the 18 observations [20]. Cases with <99.75% mortality on pyramids include *Helicoverpa zea* and *Helicoverpa armigera* on Cry1Ac + Cry2Ab cotton and the sugarcane borer, *Diatraea saccharalis,* on Cry1A.105 + Cry2Ab + Cry1Fa corn. These data indicate that mortality of susceptible insects on pyramids may often be to too low for pyramids to be most effective. Across 18 cases, a significant negative association occurred between survival of susceptible insects on pyramids and RKF, showing that redundant killing generally declines as survival of susceptible insects on pyramids increases [20].

No cross-resistance between toxins in a pyramid

Cross-resistance occurs when selection for resistance to a toxin causes resistance to a second toxin [28]. Strong cross-resistance between toxins reduces redundant killing because individuals resistant to one toxin can also survive exposure to one or more other toxins in the pyramid. However, weak cross-resistance reduces redundant killing only for insects that do not have high inherent susceptibility to the toxins in a pyramid. In such cases where the concentration of each toxin substantially exceeds what is needed to kill susceptible insects, the slight decrease in their susceptibility caused by weak cross-resistance is not sufficient to increase their survival on the pyramid [33]. Thus, weak cross-resistance in such pests is not expected to accelerate evolution of resistance to pyramids. By contrast, weak cross-resistance is expected to accelerate evolution of resistance in pests with inherently low susceptibility to Bt toxins [20, 26, 31, 34]. In these cases, some susceptible insects already survive on pyramids, implying that weak cross-resistance is expected to increase survival on pyramids [20, 26, 31, 34].

It is generally agreed that cross-resistance is less likely between toxins that differ markedly in structure and target sites [35]. Nevertheless, an analysis of 80 cases involving 10 major pests and 7 sets of Bt toxins showed that cross-resistance between toxins used in pyramids is pervasive [20]. To avoid between-strain differences that were unrelated to resistance, this analysis considered only related pairs of pest strains in which one strain was selected with a toxin in the laboratory and the other was not. For each pair of strains, cross-resistance ratios were calculated for toxins not used for selection, by dividing the LC_{50} or IC_{50} (concentration killing or inhibiting growth of 50% of tested insects, respectively) for the selected strain by the LC_{50} or IC_{50} of the unselected strain. A ratio of 1 is expected without cross-resistance and >1 with cross-resistance. It was >1 for 75 cases and <1 for only 5 cases [20]. Furthermore, for 5 of the seven sets of toxins examined (Cry1Aa and Cry1Ab; Cry1Aa and Cry1Ac; Cry1Ab and Cry1Ac; Cry1Ab or Cry1Ac and Cry1Fa; Cry1Ac or Cry1Ab and Cry2Ab), the average cross-resistance ratio was significantly greater than 1, demonstrating significant cross-resistance between toxins in these sets [20]. For two toxin sets (Cry1Ac and Cry2Aa; Cry1Ac and Vip3Aa), the average resistance ratio was greater than 1 but statistical significance was marginal. In both of these cases, a subsequent analysis based on more observations showed significant cross-resistance [34]. Overall, the data indicate that cross-resistance is pervasive between toxins currently used in pyramids. This cross-resistance is sometimes weak and thus likely to reduce durability of pyramids only against pests that have low inherent susceptibility to the Bt toxins in the pyramids.

Recent analyses suggest that understanding the mechanism of resistance and considering the implications for cross-resistance can help to improve the combinations of toxins chosen for pyramids [20, 34]. Although diverse mechanisms of resistance to Bt toxins are known, the most common and potent type involves changes in receptor proteins that reduce the binding of Bt toxins to larval midguts [35-37]. Cry toxins bind to several proteins in larval midguts including cadherins, aminopeptidases, and alkaline phosphatase [38]. Mutations in or reduced transcription of these binding proteins are associated with resistance to Cry toxins in many insects [37-39]. Alternative splicing and mis-splicing of cadherin RNA is also associated with resistance [40]. Resistance to Cry1 and Cry2 toxins is associated with mutations in ATP-binding cassette (ABC) transporter proteins in at least eight species of Lepidoptera [41-45]. It has been hypothesized, but not yet directly demonstrated, that these ABC transporter proteins also bind Cry toxins [41,42].

In general, cross-resistance is expected to be stronger between toxins that are more similar. In particular, among the Bt toxins used in transgenic crops, cross-resistance is likely to be stronger among the Cry1, Cry2 and Cry3 toxins that share a similar three-domain structure than between this set of toxins and those that do not have a three-domain structure such as Vip3Aa and Cry34/35Ab (Figure 2).

A more specific hypothesis is that cross-resistance is associated with similarity between domain II of toxins, because this domain plays a key role in binding of toxins to larval midgut receptors and altered binding is the most important mechanism of resistance [20, 46, 47]. This hypothesis was spurred by responses of a resistant strain of diamondback moth, *Plutella xylostella*, to 14 Cry1 and Cry2 toxins [46]. In this case and a recent study of *H. zea*, the association between cross-resistance and amino acid sequence similarity was stronger for domain II than domains I or III [34, 46]. A recent analysis of 80 cases evaluating cross-resistance in 10 major pests to seven sets of Bt toxins confirms this pattern and shows that amino acid sequence similarity of domain II, but not domain I and III, is associated with cross-resistance [20]. For example in *D. v. virgifera*, cross-resistance was strong between Cry3Bb and mCry3Aa [48, 49], which have 83% amino acid sequence similarity in domain II [20]. By contrast, neither Cry3Bb nor mCry3Aa have structural homology with Cry34/35Ab (Figure 2), and cross-resistance was much weaker between Cry3Bb or mCry3Aa and Cry34/35Ab [48, 49]. The low but statistically significant cross-resistance seen between pairs of toxins that are not structurally similar and are unlikely to share high-affinity binding sites implies that mechanisms other than reduced binding can cause weak cross-resistance between unrelated Bt toxins [20, 34, 50].

Pyramids are not grown concurrently with plants that produce only one of the toxins in the pyramid

Results from mathematical models as well as from laboratory and greenhouse experiments indicate that resistance to pyramids evolves faster when single-toxin plants that produce one of the toxins in the pyramid co-occur with two-toxin plants [21, 51, 52]. This happens because single-toxin crops act as stepping stones for resistance to pyramids by selecting for resistance to one of the toxins in the pyramid. For insects resistant to one toxin in a two-toxin plant, the plant does not act as a pyramid. Therefore, pyramids are most durable when they precede or rapidly

replace single-toxin crops and are introduced when pest populations are still susceptible to all of the toxins in the pyramid.

For example, replacement of Cry1Ac cotton by Cry1Ac + Cry2Ab cotton was accomplished in a single year (2004) in Australia [53] and the percentage of resistant individuals remained <1% for each toxin in both of the key target pests *Helicoverpa armigera* and *Helicoverpa punctigera* more than a decade after the pyramid was introduced [54]. In contrast, replacement of Cry1Ac cotton by Cry1Ac + Cry2Ab or Cry1Ac + Cry1Fa cotton took eight years in the U. S. [26] and was started after practical field-evolved resistance to Cry1Ac had occurred in the related pest *H. zea* [19, 55]. In less than 3 years after the pyramid was introduced, the percentage of individuals resistant to Cry2Ab was >50% in some populations of *H. zea* [19]. In India, replacement of Cry1Ac cotton by Cry1Ac + Cry2Ab cotton was still not completed after nine years [27], yielding a high risk that populations of pink bollworm (*Pectinophora gossypiella)* already resistant to Cry1Ac would rapidly evolve resistance to Cry2Ab [56]. Replacement of Cry1Ac cotton by pyramided Bt cotton has not been initiated in China, despite the small yet significant increase in *H. armigera* resistance to Cry1Ac between 2002 and 2013 [23 ,57].

Commercial release of pyramided cotton with three toxins (Cry1Ac + Cry2Ab + Vip3Aa) is anticipated for 2016 in Australia and the U.S. [20, 58]. This three-toxin pyramid is expected to be especially durable in Australia, where the frequency of resistance to all three toxins is relatively low in *H. armigera* and *H. punctigera* [54]. However, in some U.S. populations of *H. zea* already resistant to Cry1Ac and Cry2Ab, the risk of resistance to this three-toxin cotton is high because it will function as a single-toxin crop.

Because of cross-resistance between closely related Bt toxins, concurrent culture of a pyramid with a single-toxin crop that produces a toxin similar to one of the toxins in the pyramid can also accelerate evolution of resistance to the pyramid. For example, a strain of fall armyworm, *Spodoptera frugiperda,* that had field-evolved practical resistance to Cry1Fa corn rapidly evolved resistance to a pyramid of Cry1A.105 + Cry2Ab corn when exposed to this pyramid in the laboratory [52]. Cry1Fa and Cry1A.105 are closely related and cross-resistance to Cry1A.105 caused by resistance to Cry1Fa in *S. frugiperda* probably accelerated evolution of resistance to this pyramid [52]. The risk of rapid *S. frugiperda* resistance to Cry1A.105 +

Cry2Ab corn in Brazil is also high, because this pyramid is being used remedially to counter resistance to Cry1Fa [52].

Single-toxin corn hybrids targeting lepidopterans, coleopterans, or both are presently used concurrently with pyramided Bt corn hybrids in the U.S. (Table 2). Furthermore, some of these pyramids targeting lepidopteran pests are effectively single-toxin crops against important corn pests. For example, Cry1Ab and Vip3Aa corn is an effective pyramid for ear protection against *H. zea,* but functions as a single-toxin crop for ear protection against European corn borer, *Ostinia nubilalis* (which is not highly susceptible to Vip3Aa), or a single-toxin crop for whorl protection against *S. frugiperda* (which is not highly susceptible to Cry1Ab) [59, 60]. The evolution of practical resistance to mCry3Aa and Cry3Bb corn in *D. v. virgifera* in Iowa and Nebraska [48, 49] implies that all pyramids targeting this pest (Table 2) function as single-toxin crops in some regions of these states. Field-evolved practical resistance to single-toxin corn was also documented in other key pests in the U.S. (i.e., Cry1Ab corn in *H. zea* and Cry1Fa corn in *S. frugiperda*) [61, 62], which are targeted by several types of pyramided corn and cotton producing one of these toxins or a closely related toxin. Rapid phase-out of corn hybrids that function as single-toxin crops against lepidopteran and coleopteran pests should be a priority to sustain effectiveness of Bt crops in the U.S. and elsewhere.

Seed mixtures of Bt and non-Bt crops

The most effective spatial configuration of refuge plants for delaying resistance remains controversial. "Structured refuges," which are blocks of non-Bt plants grown near blocks of Bt plants, have been used extensively since 1996 in the U.S. [24]. Starting in 2010, random mixtures of Bt and non-Bt seeds (Figure 1) have been planted to manage resistance to pyramided corn [63]. Seed mixtures provide several advantages, including reduced problems with farmer non-compliance with block refuge requirements [64]. However, mathematical models show that seed mixtures can significantly accelerate resistance relative to block refuges when larvae move extensively between plants [65, 66]. Specifically, seed mixtures of non-Bt plants with single-toxin crops or pyramids can accelerate resistance by reducing survival of susceptible insects and effective refuge size; or by increasing survival of heterozygotes relative to susceptible homozygotes, thereby increasing the dominance of resistance in seed mixtures relative to blocks of Bt crops.

Laboratory and greenhouse experiments with single-toxin plants demonstrate that increased dominance of resistance in seed mixtures is most likely in pests with low inherent susceptibility to Bt toxins. In a model system involving *H. zea,* which has relatively low inherent susceptibility to Cry1Ac cotton [26], the dominance of resistance was significantly higher in a seed mixture relative to a homogeneous block of Cry1Ac cotton, because survival of heterozygotes relative to susceptible individuals increased more in the seed mixture than in the block of Bt cotton [67]. In contrast, results from experiments with two pests (*P. gossypiella* and *P. xylostella*) that have relatively high inherent susceptibility to Cry1Ac suggest that the opportunity for individual larvae to eat both non-Bt and Bt plant tissues did not increase the dominance of resistance [68, 69]. Pollen-mediated gene flow between Bt and non-Bt cotton in the field yields bolls with various proportions of Bt and non-Bt seeds [70]. However, in the seed-feeding pest *P. gossypiella*, the dominance of resistance did not vary significantly when Cry1Ac-susceptible, heterozygous, and Cry1Ac-resistant larvae fed in artificial bolls containing different proportions of Bt and non-Bt seeds [69]. In a selection experiment involving a model system with *P. xylostella* and non-commercial Cry1Ac broccoli, the percentage of larvae susceptible to Cry1Ac at the end of the experiment was not lower in seed mixture plots compared with plots containing separate blocks of Bt and non-Bt plants [68]. These results indicating that seed mixtures did not accelerate the evolution of resistance also suggest that seed mixtures did not increase the dominance of resistance. Empirical data are lacking to evaluate effects of seed mixtures of pyramided crops on the dominance of resistance.

Even without larval movement between plants, pollen-mediated gene flow could accelerate evolution of resistance in seed mixtures relative to structured refuges for insects that eat corn kernels (e.g., *H. armigera, H. zea*, *S. frugiperda*). Gene flow between Bt and non-Bt corn in seed mixtures produces a mosaic of Bt and non-Bt kernels in ears of non-Bt corn plants [71,72]. The Bt toxins in kernels of refuge plants within seed mixtures could accelerate resistance by killing susceptible larvae and reducing effective refuge size [72], increasing the dominance of resistance, or both. Empirical data are lacking to evaluate effects of gene flow on resistance evolution in seed mixtures.

Conclusions

Here we show that some of the key conditions favoring durability of Bt crops frequently are not met, especially for pests with inherently low susceptibility to Bt toxins. As the use of pyramids continue to increase and expand, it will be increasingly important to develop resistance management strategies that consider all key factors affecting resistance in all key pests targeted by Bt crops within a region. Although new Bt toxins and ways of killing pests will undoubtedly become available in the future, about 12 years is currently needed to develop novel insecticidal transgenic crops in the U.S. [73]. The refuge strategy has been successful for delaying resistance to Bt crops in pests with high susceptibility to Bt toxins [19, 74]. However, the rapid evolution of resistance recently observed in pests with low susceptibility to Bt toxins such as *S. frugiperda* and *D. v. virgifera* (Table 1) indicates that some pests could rapidly overcome most or all Bt crops available to control them. To sustain effectiveness of Bt crops against such problematic pests, refuge size will need to be increased and refuge strategies enhanced with other pest management tactics (Box 2) [26, 34, 75, 76]. The last 20 years have confirmed that insects are champions of adaptive evolution. We hope that the development of innovative resistance management strategies will continue to sustain benefits provided by transgenic insecticidal crops for the next 20 years.

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Glossary

Bt crop: a crop genetically engineered to produce one or more insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt).

Field-evolved resistance: a genetically based decrease in susceptibility of a population to a toxin caused by exposure to the toxin in the field.

Fitness cost: a trade-off in which alleles conferring resistance to a toxin reduce fitness in environments lacking the toxin.

Incomplete resistance: resistant individuals have lower fitness on the Bt crop than on the corresponding non-Bt crop.

Practical resistance: field-evolved resistance that reduces Bt crop efficacy and has practical consequences for pest control.

Pyramided transgenic crop: a crop genetically engineered to produce two or more distinct toxins that kill the same pest.

Redundant killing: insects resistant to one toxin produced by a pyramided Bt plant are killed by another toxin produced by that plant

Refuge: host plants that do not produce Bt toxins and thus promote survival of pests that are not resistant to Bt toxins.

Seed mixture: a random mixture of seeds of Bt and non-Bt plants of the same crop (also referred to as "refuge-in-a-bag" or RIB) used to delay field-evolved resistance in pests.

Stacked transgenic crop: a crop genetically engineered for protection against insects and one or more herbicides.

Structured refuge: non-Bt crops planted contiguously in blocks or entire fields to delay field-evolved resistance in insect pests.

Box 1. Categories and patterns of field-evolved resistance to Bt crops.

Recognizing that resistance is not "all or none" and that various levels of resistance can have a continuum of effects on pest control, five categories of field-evolved resistance to Bt crops have been described [28, 29]. All five categories entail a statistically significant, genetically based decrease in susceptibility in field populations of pests, but only one category (practical resistance) indicates resistance is severe enough to generate reports of reduced pest control in the field: 1) incipient resistance: <1% resistant individuals, 2) early warning of resistance: 1% to 6% resistant individuals, 3) >6% to 50% resistant individuals, 4) >50% resistant individuals and reduced efficacy expected but not reported, and 5) practical resistance: >50% resistant individuals and reduced efficacy reported. In a recent analysis, 12 of of 27 cases examined (44%) showed no significant increase in resistance after 2 to 15 years (median = 8 years) of exposure to Bt crops [29]. Of the remaining 15 cases, three were characterized as incipient resistance, four were early warning of resistance, one was >50% resistant individuals with reduced efficacy expected but not reported, and seven demonstrated practical resistance. All seven cases of practical resistance involved resistance to single-toxin crops (Table 1). Field-evolved resistance to Cry2Ab, which has been used only in combination with one or more other Bt toxins, has been documented in populations of two closely related species (*Helicoverpa punctigera* and *Helicoverpa zea*) that were exposed extensively to a Bt cotton pyramid of Cry1Ac and Cry2Ab, but neither of these cases has been categorized as practical resistance [19, 55, 77].

Box 2. Can seed mixtures delay rootworm resistance to Bt corn?

The conditions for *Diabrotica v. virgifera* and Bt corn deviate from conditions favoring durability in the following ways: 1) alleles conferring resistance are not rare; 2) resistance is not recessive; 3) fitness costs appear minimal; 4) cross-resistance occurs between some toxins used in pyramids, and 5) pyramids are grown concurrently with plants that produce one of the toxins in the pyramid (see text and Table 2) [76, 78].

Field-evolved practical resistance of *D. v. virgifera* to single-toxin Bt corn producing either Cry3Bb or mCry3Aa has been documented in Iowa and Nebraska [48, 49]. This is not surprising because *D. v. virgifera* rapidly evolved resistance to Bt corn producing either Cry3Bb or mCry3Aa in laboratory and greenhouse selection experiments [76, 78]. Because analogous experiments show rapid evolution of resistance to Bt corn producing Cry34/35Ab [76, 78], the risk of evolution of resistance to Bt corn pyramids producing either Cry3Bb + Cry34/35Ab or mCry3Aa + Cry34/35Ab is high where this pest has already evolved resistance to Cry3Bb and mCry3Aa. Cry3Bb and mCry3Aa are 83% similar in domain II and cross-resistance occurs between them [20, 48]. Furthermore, amino acid sequence similarity in domain II between mCry3A and eCry3.1Ab is 100% [20], indicating that cross-resistance between them is likely. Accordingly, the risk of evolution of resistance to mCry3A + eCry3.1Ab corn is also high.

Extensive larval movement between Bt and non-Bt plants occurred when *D. v. virgifera* were exposed to seed mixtures of non-Bt corn and a Bt corn pyramid producing Cry3Bb + Cry34/35Ab [64, 79]. Larval movement in seed mixtures from Bt to non-Bt plants increased survival of susceptible larvae relative to their survival in blocks of Bt plants [64, 79]. Conversely, larval movement from non-Bt to Bt plants reduced survival of susceptible individuals relative to their survival on blocks on non-Bt plants [64, 79]. The evolution of resistance could be accelerated in seed mixtures if individuals with one or more resistance alleles have lower mortality than susceptible individuals when larvae move from Bt to non-Bt plants or from non-Bt to Bt plants. Because the risk of resistance to pyramids in seed mixtures is high in *D. v. virgifera*, integrating crop rotation with use of seed mixtures in regions where this pest remains susceptible to crop rotations could enhance resistance management [78, 80].

Table 1. Seven cases of field-evolved practical resistance to single-toxin Bt crops [19, 29].

Insect	**Bt crop**	**Toxin**	**Country**	**Durability (years)** [a]	**Initial detection** [b]
Helicoverpa zea	Cotton	Cry1Ac	USA	6	2002
Busseola fusca	Corn	Cry1Ab	South Africa	8	2006
Spodoptera frugiperda	Corn	Cry1Fa	USA	3	2008
Pectinophora gossypiella	Cotton	Cry1Ac	India	6	2009
Diabrotica virgifera virgifera	Corn	Cry3Bb	USA	7	2009
Diabrotica v. virgifera	Corn	mCry3A	USA	4	2011
S. frugiperda	Corn	Cry1Fa	Brazil	2	2011

[a] Years elapsed in the region studied between the first year of commercial use and the first year of field sampling that yielded evidence of practical resistance

[b] First year of field sampling that provided evidence of practical resistance; publication of this evidence often occurred several years later. For example, evidence of *S. frugiperda* resistance to Cry1Fa in Brazil was published first in 2014 based on bioassay data from progeny of insects sampled from the field in 2011 [81].

Table 2. Twenty-one sets of one to five Bt toxins produced by Bt corn hybrids used in the United States [82-84].

Bt toxin(s) [a]	**Single toxin against Lepidoptera**	**Single toxin against Coleoptera**	**Pyramid against Lepidoptera** [b]	**Pyramid against Coleoptera**
Cry1Ab	X			
Cry1Fa	X			
Cry3Bb		X		
Cry34/35Ab		X		
mCry3Aa		X		
Cry1Ab+Cry3Bb [c]	X	X		
Cry1Ab+mCry3Aa [c]	X	X		
Cry1Fa+Cry34/35Bb [c]	X	X		
Cry1Fa+mCry3Aa [c]	X	X		
Cry1A.105+Cry2Ab+Cry3Bb [d]		X	X	
Cry1Ab+Cry1Fa+Cry34/35Bb [d]		X	X	
Cry1Ab+Vip3Aa+mCry3Aa [d]		X	X	
Cry1Ab+Cry1Fa+mCry3Aa+eCry3.1Ab [e]		X	X	
Cry1Ab+Cry1Fa+Vip3Aa+mCry3Aa+eCry3.1Ab [e]		X	X	
Cry1A.105+Cry2Ab			X	
Cry1Ab+Cry1Fa			X	
Cry1Ab+Vip3Aa			X	
Cry1Ab+Cry1Fa+Vip3Aa			X	
Cry1A.105+Cry1Fa+Cry2Ab			X	
Cry1Ab+Cry1Fa+mCry3Aa+Cry34/35Ab [e]			X	X
Cry1A.105+Cry1Fa+Cry2Ab+Cry3Bb+Cry34/35Ab [e]			X	X

[a] Relative to using pyramids alone, resistance in a particular pest evolves faster when plants that produce only one toxin effective against that pest are planted concurrently with crops that are pyramided against that pest.

[b] Some plants producing two toxins are not pyramided against particular Lepidoptera when only one of the toxins is active against those species

[c] One toxin targets Lepidoptera and the other toxin targets Coleoptera

[d] Two toxins from the Cry1, Cry2, or Vip3 families target Lepidoptera and the other toxin targets Coleoptera

[e] Because resistance to Cry3Bb and mCry3Aa has occurred in *D. v. virgifera* in some regions of the United States, plants producing these two toxins do not act as pyramids against *D. v. virgifera* in those regions

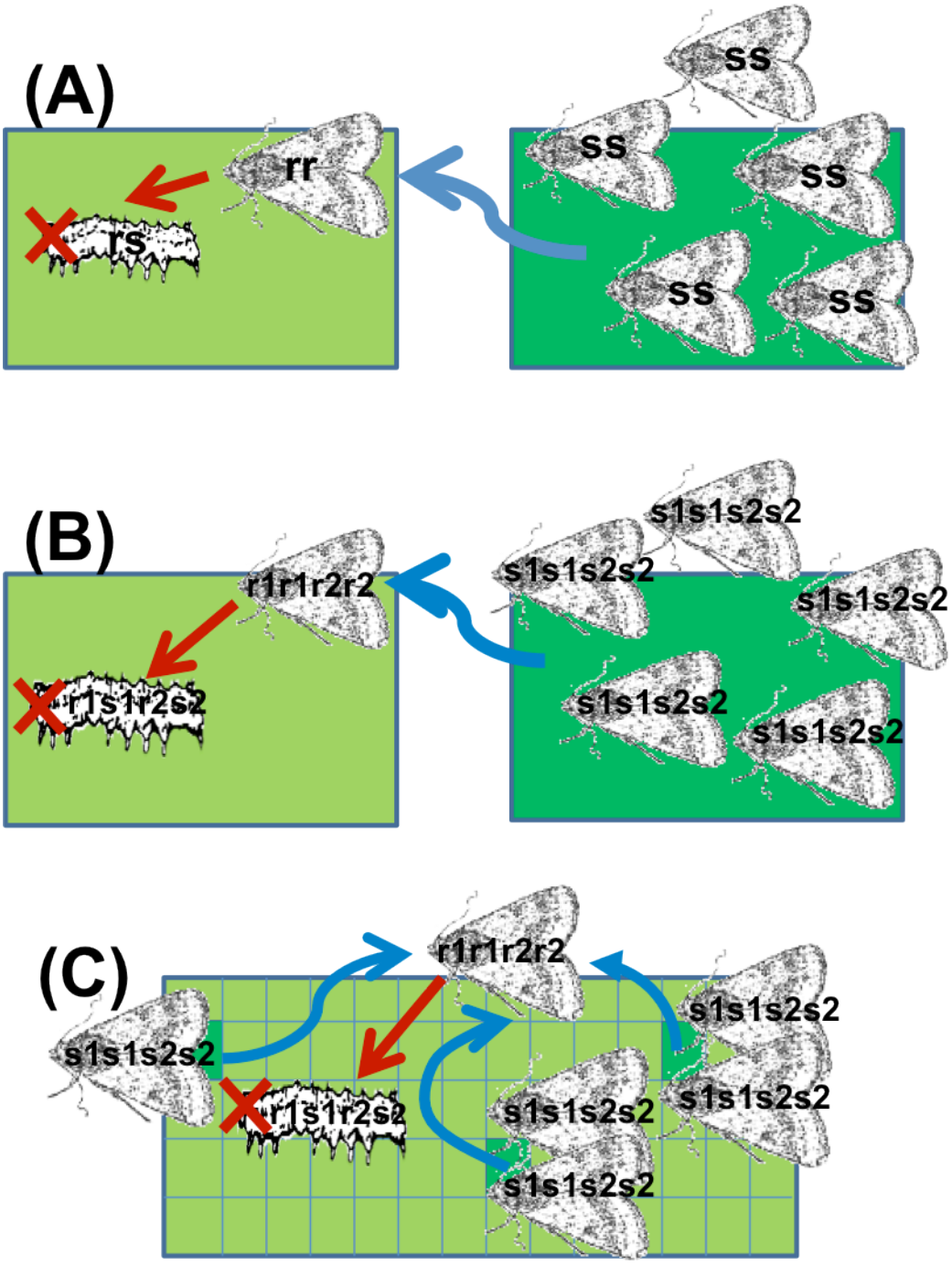

Figure 1. The refuge strategy for delaying insect resistance to (A) single-toxin or (B) pyramided Bt crops with structured refuges, and (C) seed mixtures of pyramided Bt crops and non-Bt crops. In (A) and (B), each field contains either Bt plants (light green) or non-Bt plants (dark green). In (C), Bt plants (light green squares) and non-Bt plants (dark green squares) are randomly distributed within each field. Under ideal conditions, alleles conferring resistance (r) to Bt crops

are rare and resistance is recessive so that heterozygotes carrying one allele for resistance and another for susceptibility (s) are killed by Bt crops. In principle, the relatively abundant homozygous susceptible moths (ss in A and s1s1s2s2 in B and C) in refuges mate with the rare homozygous resistant moths (rr in A and r1r1r2r2 in B and C; pointed to by blue arrows) surviving on Bt crops. The resulting offspring (caterpillars pointed to by red arrows) are heterozygous for resistance (rs in A and r1s1r2s2 in B and C) and are killed by Bt crops (red crosses), which delays evolution of resistance.

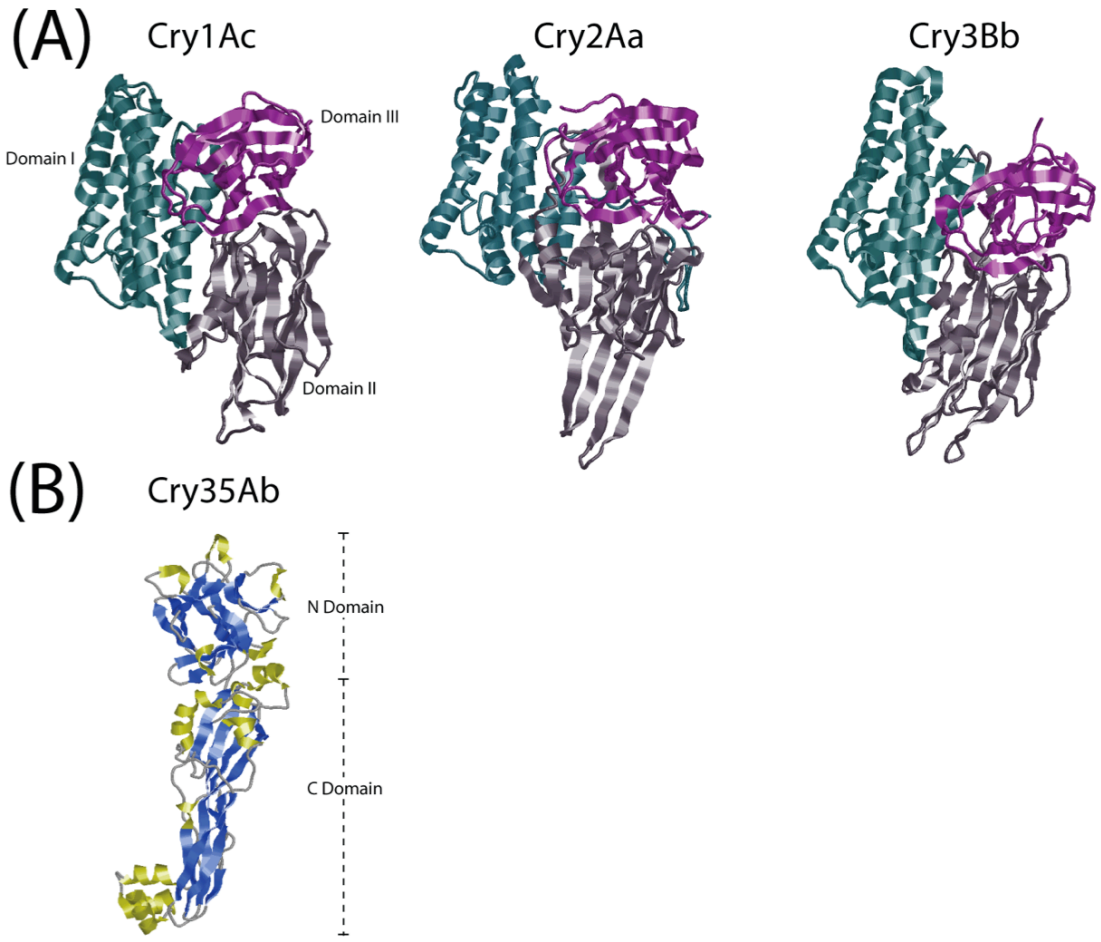

Figure 2. (A) X-ray crystal structures of the three-domain crystal proteins Cry1Ac (PDB 4ARY), Cry2Aa (PDB 1I5P), and Cry3Bb (PDB 1JI6) and (B) the Bin-like toxin Cry35Ab (PDB 4JP0). (A) Although the specificity of insecticidal activity for the three-domain toxins differs

dramatically (Cry1Ac kills some Lepidoptera, Cry2Aa kills some Lepidoptera and Diptera, and Cry3Bb targets some Coleoptera) (Table 2), their three-dimensional structures share considerable similarity. Domain I (shown in blue-green) is comprised of a seven α-helix bundle that inserts into the insect midgut membrane to form a pore. Domain II (shown in grey) is a β-prism of three anti-parallel β-sheets involved in binding to midgut receptors primarily through the exposed loops. Domain III (shown in purple) has two anti-parallel β-sheets and contributes to receptor binding. (B) Bin-like proteins are β-forming toxins that share similarity with the aerolysin-type pore-forming toxins but differ structurally from the three-domain Cry proteins. The protein Cry35Ab (PDB 4JP0) has two domains, an amino-terminal β-trefoil domain (N Domain) and the carboxyl-terminal domain with extended antiparallel β-sheets (C Domain) similar to aerolysin folds. β-sheets are shown in blue and α-helices in yellow. Bin-like toxins often require the formation of binary interactions with other protein partners for toxicity (for example, Cry35Ab requires Cry34Ab to form the Cry34/35Ab complex that is toxic to some coleopterans) [85].