Review

# Crop improvement using small RNAs: applications and predictive ecological risk assessments

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**Crops can be modified by engineering novel RNA interference (RNAi) pathways that create small RNA molecules to alter gene expression in crops or plant pests. RNAi can generate new crop quality traits or provide protection against insects, nematodes and pathogens without introducing new proteins into food and feed products. As a result, stakeholders and regulators need to construct credible ecological risk assessments (ERAs) that characterize potential exposure pathways and hazards for RNAi crops, including off-target effects, non-target effects and impacts from genetic mutations and polymorphisms. New methods are needed to identify RNAi crops and measure the environmental persistence of small RNAs. With some modifications, it seems likely that current ERA frameworks can be applied to most crops engineered through RNAi.**

## Introduction to RNA-mediated crop improvement

Countries around the world have long acted on the belief that public and private investment in basic and applied plant sciences will help to overcome challenges in crop production. Today, that long-term vision of crop improvement often combines traditional plant breeding with innovations made possible by biotechnology. This paper examines the potential for crop improvement through the expression of novel small RNAs and explores the implications of engineered RNA interference (RNAi) for predictive ecological risk assessment (ERA) and regulation of genetically engineered (GE) crops.

The relatively recent discovery of RNAi has supported a major paradigm shift from "one gene, one protein" to the concept that non-coding DNA can have profound effects in cells and organisms (Box 1). In the same year that Mello and colleagues [1] reported RNAi in *Caenorhabditis elegans*, RNAi was described in plants by Waterhouse *et al.* [2] for experiments that produced virus-resistant tobacco. Over the last decade, numerous papers have contributed to the view that RNAi is evolutionarily conserved in the plant kingdom and has many diverse functions [3–8].

Our understanding of RNAi has emerged from two areas of plant science, experiments creating transgenic plants and research into virus resistance [4,6–10]. In the late 1980s, scientists observed that some transgenic plants did not express the protein expected from a transgene linked to a strong promoter sequence. In some cases, the transgene was able to silence expression of a homologous native gene. Scientists called these unexpected results gene silencing, post-transcriptional gene silencing (PTGS) or co-suppression. Unexpected gene silencing from recombinant DNA in the antisense direction was called antisense technology. Around the same time, researchers discovered that expression of recombinant viral coat protein genes could confer resistance to the virus from which the coat protein was taken [7,11]. Some of the earliest GE crops

### Glossary

**Antisense technology**: gene silencing is caused by insertion of a DNA sequence in the plant's nuclear genome in the reverse (antisense) direction. Antisense RNA interacts with sense mRNA, thus blocking protein synthesis.
**Co-suppression**: early term used by plant biologists to describe RNA-mediated interference with transgene expression.
**Double-stranded RNA (dsRNA)**: ribonucleotide strand containing RNA sequences in the sense and antisense orientations that completely or partially complement each other. dsRNA may form hairpin RNA.
**Hairpin RNA (hpRNA)**: ribonucleotide strand with complementary sense and antisense sequences that folds to produce double-stranded RNA with a loop at one end. Also called a short hairpin or small hairpin RNA (shRNA).
**Host-delivered RNA interference (HD-RNAi)**: engineering of RNAi in crop plants so that small RNAs will be ingested by pest organisms (pathogens, nematodes, insects) and degrade targeted mRNAs and silence essential genes in the pest.
**mRNA**: ribonucleotide strand produced by transcription and subsequent processing. The mRNA codes for a polypeptide (e.g. structural proteins, enzymes).
**Micro RNA (miRNA)**: single-stranded short RNA molecules (21–23 base pairs) that regulate gene expression in plants and other eukaryotes. The functional miRNA is derived from primary micro RNAs (pri-miRNA). In general, miRNA differ from small interfering RNA (siRNA) in that they are usually produced from dsRNA. miRNAs are also defined by having sequences that are only partially complementary to the target mRNAs. Some organisms have the capacity to duplicate miRNA to amplify the RNAi signal and to create systemic RNAi effects.
**Non-target effects**: unintended impacts (generally negative effects) of GE crops on species (e.g. beneficial insects) on direct or indirect exposure to the GE crop.
**Off-target effects**: unintended and/or pleiotropic effects that occur as a result of sequence homology between novel, small RNAs in the GE crop and mRNA in the host crop plant or organisms in the environment. Examples of off-target effects include gene silencing that leads to unexpected pollen lethality, and unintended gene silencing in beneficial insects exposed to the crop plant.
**Post-transcriptional gene silencing (PTGS)**: term used to describe silencing of transgene expression at the mRNA level.
**RNA interference (RNAi)**: inclusive term for the action of small interfering RNAs and microRNAs resulting in gene silencing through cleavage of mRNAs and blockage of protein synthesis (Box 1).
**RNA-dependent RNA polymerase (RdRp, RDR)**: class of RNA polymerases that use single-stranded RNA as a template to produce many additional copies of small RNA molecules, thereby amplifying RNAi.
**Small RNAs/small-interfering RNA (siRNA)/short silencing RNA (sRNA)**: short, single-stranded, non-coding RNA molecule involved in RNAi. siRNA is typically 21–24 nucleotides in length (Box 1).

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**Box 1. Overview of RNAi in plants**

RNAi alters plant growth and development by stopping mRNA molecules from serving as the template for protein synthesis. RNAi can be conducted using two types of single-stranded RNA molecules, siRNA and miRNA. RNAi decreases or eliminates gene expression by cleaving targeted mRNA molecules or by interfering with translation. A third mechanism of action is small RNA-directed DNA methylation (RdDM), creating epigenetic effects (heritable modifications of DNA structure) through *de novo* methylation of cytosine bases in DNA regions homologous to small RNA [60–62]. More than two million small RNA molecules have been identified in *Arabidopsis*, many of which are coded in genomic regions previously labeled as non-coding or junk DNA [63]. A basic outline of RNAi is provided here, but other papers offer more details and illustrations [4,6–9].

(i) Transcription occurs when RNA polymerase II enzyme reads a non-coding DNA sequence and produces a complementary strand of RNA in the plant cell nucleus.
(ii) In many cases, the newly synthesized RNA strand will contain stretches of ribonucleotides that complement each other (sense and antisense sequences). When this occurs, the RNA strand will fold back on itself and form a double-stranded RNA (dsRNA) molecule with a hairpin loop at one end called hairpin RNA (hpRNA) or pre-micro RNA.
(iii) A multi-protein Dicer complex (Dicer-like enzymes, RNAase III enzymes) clips the dsRNA to produce shorter sections of dsRNA of approximately 21–24 bp in length. These short RNA duplexes (sense and antisense strands) are unwound to produce a single guide strand. It is generally believed that this occurs in the nucleus, followed by export to the cytoplasm.
(iv) In the cytoplasm, the siRNA or miRNA guide strand interacts with the RNA-induced silencing complex (RISC) including the protein Argonaute (Ago). The RISC helps the guide strand to find its target mRNA with complete or partial sequence complementarity. miRNA typically has slight mismatches with the target mRNA, whereas siRNA molecules fully complement their target mRNA. Target sequences are usually in the coding region of the mRNA, but can occur in the 3′ untranslated region (3′UTR).
(v) RISC cleaves the target mRNA into smaller pieces that no longer function as templates for protein synthesis. In some cases, small RNA molecules and specific Argonaute proteins can inhibit translation without mRNA cleavage [64].
(vi) Plants are among the eukaryotes that can amplify the RNAi effect using RNA-dependent RNA polymerase (RdRp) enzymes to duplicate siRNA molecules.
(vii) Plants have systemic RNAi systems that move siRNA molecules between neighboring cells via plasmodesmata or through the phloem [3]. Intercellular movement or phloem transport is not required for RNAi.

grown in the USA used the action of small RNAs, even though this cellular mechanism was not completely understood (Table 1). For example, FlavrSavr tomatoes showed delayed fruit ripening through suppression of the polygalacturonase enzyme [12]. GE papaya trees expressing papaya ring spot coat protein genes were planted for virus resistance [13]. Today, researchers are engineering a variety of crops to produce small RNAs that will silence essential genes in insects, nematodes and pathogens, an approach called host-delivered RNAi (HD-RNAi). Because biotechnology companies protect much of their research as confidential business information, it is impossible to accurately assess the current level of innovation, but this section provides a sense of the scope and direction of RNAi crops.

### *Crop quality traits*

It is well accepted that RNAi can improve the nutritive value of crops (e.g. amino acids, fatty acids, fiber), eliminate allergenic compounds, create male sterility for crop breeding, decrease toxic compounds and modify many other traits [5,6,14,15]. Sunilkumar *et al.* [16] reduced

**Table 1. GE crops in the USA using RNAi[a]**

| Crop plant | Trait type | Applicant | Approval | Mechanism of action[b] |
|---|---|---|---|---|
| Papaya | Virus resistance | University of Florida | Pending | VCPG from PRSV virus |
| Plum tree | Virus resistance | USDA-ARS | Pending | VCPG from plum pox virus |
| Tobacco | Product quality | Vector Tobacco | 2001 | Nicotine reduced through gene silencing in biosynthetic pathway |
| Potato | Virus resistance | Monsanto | 2000 | VCPG for PLRV virus |
| Potato | Virus resistance | Monsanto | 1998 | VCPG for PVY virus |
| Potato | Virus resistance | Monsanto | 1998 | VCPG for PVY virus |
| Potato | Virus resistance | Monsanto | 1998 | VCPG for PLRV virus |
| Soybean | Oil quality | DuPont | 1997 | Silencing of *GmFAD2-1* gene to increase oleic acid content |
| Tomato | Fruit quality | Calgene | 1996 | Silencing of polygalacturonase gene |
| Papaya | Virus resistance | Cornell University | 1996 | VCPG from PRSV virus [13] |
| Squash | Virus resistance | Asgrow | 1996 | VCPG from CMV, WMMV2 and ZYMV viruses |
| Tomato | Fruit quality | Calgene | 1995 | Silencing of polygalacturonase gene |
| Tomato | Fruit quality | Calgene | 1995 | Silencing of polygalacturonase gene |
| Tomato | Fruit quality | Zeneca/Petroseed | 1995 | Silencing of polygalacturonase gene |
| Tomato | Fruit quality | Calgene | 1994 | Silencing of polygalacturonase gene |
| Tomato | Fruit quality | DNA Plant Tech | 1995 | Silencing of amino cyclopropane carboxylic acid synthase (ACCS) involved in ethylene biosynthesis |
| Tomato | Fruit quality | Calgene | 1994 | Silencing of polygalacturonase gene |
| Squash | Virus resistance | Upjohn | 1994 | VCPG from MWV2 and ZYMV viruses |
| Tomato (Flavr Savr) | Fruit quality | Calgene | 1992 | Silencing of polygalacturonase gene involved in fruit ripening [12] |

[a]Development of these GE crops by government agencies, academic laboratories, and biotechnology companies suggests some familiarity with the technology. (http://usbiotechreg.nbii.gov, http://www.aphis.usda.gov/brs/not_reg.html).
[b]VCPG, viral coat protein gene.

the toxic terpenoid gossypol in cotton seeds and cotton oil by engineering small RNAs for the cadinene synthase gene in the gossypol biosynthesis pathway. A seed-specific promoter ensured that the gene was silenced in cotton seed, while allowing the leaves to synthesize normal terpenoid levels for protection against insects.

### *Virus resistance*

RNAi is a powerful natural pathway for virus resistance in plants [7,11,17–19]. Resistance to RNA viruses occurs through a self-perpetuating (RNA-dependent RNA polymerase) sequence-specific degradation of targeted viral mRNA. Experiments with tobacco showed that HD-RNAi could be achieved using siRNA or dsRNA molecules that complemented viral coat proteins [2,11]. HD-RNAi is used today in virus-resistant squash and papaya (Table 1). However, RNAi has not been able to provide protection from single-stranded DNA geminiviruses that cause significant damage to crops such as cassava and tomato [20,21].

### *Protection from insect pests*

Unlike plants, insects, mollusks and vertebrates seem to lack genes for the RNA-dependent RNA polymerase (RdRp) enzyme that replicates siRNA molecules and creates systemic RNAi action [22–24]. This and other technical issues initially led to pessimism about the development of HD-RNAi to deter insect pests [23]. Two recent papers have demonstrated that HD-RNAi insect resistance is possible, although its efficacy under field conditions is not yet confirmed. Baum *et al.* [25] showed that silencing of a vacuolar ATPase gene (V-type ATPase A gene) in midgut cells of western corn rootworm (WCR) led to larval mortality and stunted growth. Transgenic maize plants expressing dsRNA for WCR V-type ATPase A showed reduced feeding damage from WCR. Experiments were conducted with dsRNA for three target genes (V-ATPase A, V-ATPase E and B-tubulin) and other beetle pests. Diets with dsRNA killed southern corn rootworm and Colorado potato beetle larvae, but not cotton boll weevils. Another research group used RNAi to exploit plant secondary metabolites and the insect pathways that detoxify them. Mao *et al.* [26] showed that HD-RNAi could make cotton bollworms more susceptible to gossypol, a natural toxin in cotton plants. Researchers identified a cytochrome P450 monooxygenase (*CYP6AE14*) gene important for larval growth expressed in midgut cells with a causal relationship to gossypol tolerance. Transgenic tobacco and *Arabidopsis* producing *CYP6AE14* dsRNA were fed to larvae, successfully decreasing endogenous CYP6AE14 mRNA in the insect, stunting larval growth and increasing sensitivity to gossypol. More research is required to determine if HD-RNAi can be optimized for field conditions as an alternative to insecticides and Bt endotoxin.

### *Nematode resistance*

Recent studies suggest that HD-RNAi could offer protection against plant-parasitic nematodes [27–29]. Yadav *et al.* [30] showed that tobacco plants expressed dsRNA targeting two *Meloidogyne* (root knot) nematode genes had more than 95% resistance to *Meloidogyne incognita*. Huang *et al.* [31] showed that *Arabidopsis* plants expressing dsRNA for a gene involved in plant–parasite interaction (*16D10*) had suppressed formation of root galls by *Meloidogyne* nematodes and reduced egg production. The engineered *Arabidopsis* plants also showed some resistance to four economically important species of *Meloidogyne*. This study was the first to target a gene involved in parasitism (rather than a nematode housekeeping gene) and demonstrate resistance to more than one nematode species. It is likely that recent sequencing of the *Meloidogyne hapla* genome will reveal new targets for HD-RNAi [32].

### *Bacterial and fungal resistance*

There has been little progress in using RNAi to protect crops from bacterial and fungal pathogens. Some evidence suggests that small RNAs change their expression during pathogen attack and subsequently regulate genes involved in disease resistance pathways [33,34]. Small RNAs might silence negative regulator molecules in the plant cell under normal circumstances, but allow rapid upregulation of genes when pathogens attack. Escobar *et al.* [35] showed that silencing of two bacterial genes (*iaaM* and *ipt*) could decrease the production of crown gall tumors (*Agrobacterium tumefaciens*) to nearly zero in *Arabidopsis*, suggesting that resistance to crown gall disease could be engineered in trees and woody ornamental plants.

## Implications for ecological risk assessment (ERA)

Predictive ERAs have become an established component of the regulatory process for GE crops in many countries [36] (Box 2). Many papers and conferences have debated the utility of ERA frameworks and the best practices for implementing them [37–44]. In general, predictive risk assessment is the process by which future risks (harms, negative impacts) are estimated based on current knowledge and hypothesis-driven scientific research. Risk assessment frameworks typically involve logical steps of problem formulation, identification of potential hazards, identification of exposure pathways, risk characterization, prediction of the severity of harm (negligible, low, moderate, high) and an expression of uncertainty. The classic definition of an ecological risk is a negative impact that is the product of a hazard (a defined adverse impact on the environment) and an exposure (a mechanism or route by which the hazard is experienced). Recently, international interest has increased in using established ERA processes and frameworks such as those described in the US Environmental Protection Agency (EPA) guidelines [39,40,45]. The following discussion focuses on ERA for RNAi and HD-RNAi crops with emphasis on integration into regulatory frameworks (Box 2). Figure 1 outlines a theoretical model for risk characterization of a HD-RNAi insect-resistant crop. Table 2 compares the ERA information for two insect-resistant GE crops using either expression of the Bt toxin protein or engineered small RNAs.

Environmental risks are evaluated with regard to specific ERA endpoints that deserve protection (e.g. survival of beneficial insects) and that are relevant to the specific crop. Risk assessment endpoints can be expressed from the individual level (e.g. one individual of an endangered species) to higher organizational levels such as

**Box 2. Ecological risk assessment and regulatory frameworks for GE crops**

A considerable history of regulatory framework development predates the introduction and utilization of GE crops. This included the establishment of legal frameworks for oversight authority, as well the processes and procedures to implement regulations [65–68]. Established risk assessment processes were adapted to recognize and account for particularities in the science underlying GE crops. Before the first application for approval to release GE organisms was received, countries were thinking about assessment needs and appropriate use of scientific information to determine the level of risk associated with the production and use of GE organisms [66]. A significant challenge for risk assessors was to prepare for these new products before dossiers were submitted for regulatory approval. This challenge continues with the advancement of the science underlying risk assessment and the development of innovative technologies such as small RNAs. Regardless of the molecular mechanisms in GE crops, ERAs will most likely require detailed information on a case-by-case basis about the host crop species, the DNA sequences inserted and their mechanism of action, and the environment in which the crop will be cultivated. Although an elusive concept, familiarity has been another accepted cornerstone of risk assessment for more than a decade [69]. In future, regulators will probably be familiar with the biology and ecology of the host crop plants (e.g. maize), but some aspects specific to RNA-mediated traits (Table 2) will be less familiar. The development of systematic approaches will continue to support the risk assessment process if it takes into account prior experience, the state of the science and accepted rationales. In concert, these elements can facilitate the assessment of new products. However, even with accepted processes and frameworks in place, analysts must be familiar with the latest scientific research and methods to credibly use empirical data for risk assessments [40].

populations, communities, ecosystems and landscapes [42,45]. Mechanisms of exposure might involve direct interaction with the GE plants themselves, the protein or biochemical product of the transgene, sexually compatible plants that receive the transgene, transgenic propagules or plant parts (e.g. pollen, seeds, rhizomes, bulbs) (Figure 1). Potential hazards could include unintended effects on non-target species (e.g. mortality of beneficial insects) and the creation of problematic weeds (Figure 1). Special attention might also be paid to threatened or endangered species or to overall biodiversity, although this particular type of hazard is often difficult to predict and characterize precisely. Well-developed predictive ERAs will consider the spatial areas in which an impact would occur, the period of time during which the risk would be experienced, the reversibility of the hazards and the severity of harm to valued risk assessment endpoints. In practice, if the predictive ERA identifies some potential risks, analysts and regulators might explore ways to manage these risks through stewardship practices, containment measures or other actions.

To date, the majority of GE crops approved for commercial use contain inserted transgenes that code for bioactive proteins (e.g. Bt endotoxin, enzymes). However, some GE crops in the USA with RNA-mediated traits have already been developed and commercialized, suggesting some degree of familiarity with the technology (Table 1). With recent research opening the door to many new RNA-mediated traits, it is likely that regulators will need to assess the potential risks and benefits of an increasing number of RNAi and HD-RNAi crops proposed for experimental field trials and commercial use. In general, it seems that potential ecological risks for RNAi and HD-RNAi crops can be analyzed using the ERA framework

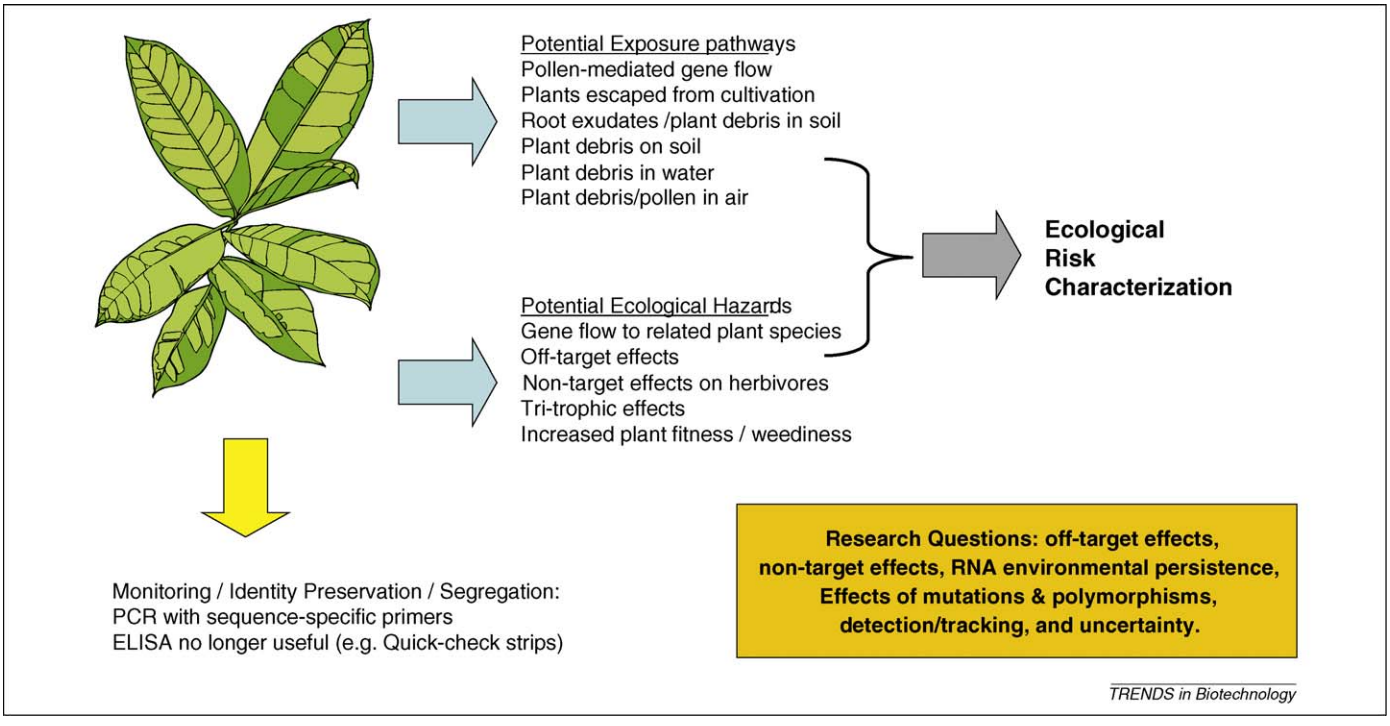


**Figure 1**. Conceptual model for ecological risk characterization and post-commercialization monitoring of a HD-RNAi crop. The theoretical GE crop expresses a transgene coding for small RNAs that will silence genes in an insect pest. The theoretical HD-RNAi crop has sexually compatible relatives in the environment with potential for gene flow. Crop species without sexually compatible relatives would not require assessment for vertical gene flow. Exposure pathways and hazards are shown as potential contributors to ecological risk. The yellow arrow indicates special considerations for HD-RNAi crop monitoring, segregation and identity preservation.

**Table 2. Ecological risk assessment: comparison of information required in an application to a regulatory agency for a GE crop expressing the *Baccillus thuringiensis* (Bt) endotoxin protein, or a HD-RNAi crop producing a small RNA with toxicity to insect pests**

| | Bt endotoxin GE crop ERA information | HD-RNAi GE crop ERA information | Challenges and questions |
|---|---|---|---|
| Molecular characterization of active molecule | Gene coding for Bt endotoxin protein | Gene coding for small RNA molecules (20–24 nucleotides) | Limited genomic databases make comparative analysis for sequence homology in non-target species difficult |
| Mode of action | Bt endotoxin protein binds to insect gut membrane receptor proteins resulting in cell lysis; action localized to insect mid-gut [70] | Multistep process involving small RNAs from crop plant and insect protein complexes leading to insect mRNA cleavage and gene silencing [9,52] | Multiple modes of action are known in *Arabidopsis*, but these are poorly understood in most crop species [9]<br>Lack of benchmarks or normalization for small RNA activity limits the ability to conduct comparative assessments [57] |
| Toxicity testing | Testing on non-target organisms, often using a tiered approach [39]<br>Allergenicity potential evaluated [36] | Toxicogenomics analysis of off-target and non-target effects<br>Testing on non-target organisms, often using a tiered approach [39]<br>Allergenicity probably not an issue | Lack of normalized genomic libraries and DNA arrays for ecotoxicological model organisms [71]<br>Validation might be needed for tiered testing of crops with RNA-mediated traits |
| Exposure assessment | Includes environmental fate estimates (crop gene flow, protein half-life in soil and water), methodology for tracking the Bt protein and its gene (lateral flow strips, ELISA, quantitative PCR), and measurement of Bt toxin distribution in plant tissues | Includes environmental fate estimates for small RNA (crop gene flow, small RNA half-life in soil and water), potential for uptake by non-target organisms, and characterization of systemic gene silencing (if present) [46] | Persistence and fate of small RNAs in ecosystems (e.g. soil, water) are largely unknown<br>Extraction and identification of small RNAs for environmental monitoring can be very difficult |
| Crop plant product sustainability | Analysis of the development of insect resistance (e.g. predictive, deterministic or stochastic models)<br>Resistance management plans<br>Transgene stability over several crop plant generations | Analysis of the development of insect resistance (e.g. predictive deterministic or stochastic models)<br>Transgene stability over several crop plant generations | Resistance development models for RNA-mediated traits have not been developed but might not be necessary to characterize risk<br>Mutation rates in genes for small RNAs can be high relative to protein-coding genes [52] |

established for other GE crops (Figure 1, Table 2). Questions about potential ecological risks are familiar to regulators and stakeholders and include the following: (i) Are there any potential hazards and exposure pathways for this GE crop? (ii) Are there likely to be significant effects on non-target organisms (e.g. beneficial insects), communities or ecosystems? (iii) Is there potential for gene flow to native or naturalized relatives that might lead to environmental consequences? (iv) Could these crops create new weeds or invasive species? (v) Is the trait stable through crop generations? Although this set of questions might function well for both protein-based GE crops and RNAi crops, it is important to recognize differences that might be relevant in an application for regulatory approval and how such information might be assessed. For example, the stability of transgenes over several plant generations is a common concern raised by regulators [36]. Genetic stability has been relevant to the long-term effectiveness of Bt crops because a loss of protective concentrations of the Bt toxin within a crop population might facilitate the evolution of insect resistance. For RNAi and HD-RNAi crops, questions and concerns about their genetic stability will have a somewhat different focus (see below).

In considering how RNAi and HD-RNAi crops might fit within the accepted ERA framework, we formulated six questions for special attention. (i) What off-target effects (defined below) could occur within the crop or in organisms consuming the crop? (ii) What non-target effects could create a hazard in the environment? (iii) How persistent are small RNA molecules in the environment? (iv) What will be the effect of mutations and polymorphisms in the crop plant and organisms consuming the crop? (v) What tools will be useful for rapidly detecting and tracking these crops and their derived products? and (vi) How should uncertainty in risk assessments be expressed? These questions are addressed below.

### *Off-target effects*

Off-target effects occur when sequence homology allows novel small RNAs to degrade mRNA for genes that are not the intended silencing targets [5,46]. Experiments with bacteria have demonstrated molecular crosstalk that decreased the expression of non-target genes [47]. If small RNAs can unexpectedly silence genes in the plant or an organism consuming the crop, questions must be asked about possible unintended effects on plant physiology and phenotypic pleiotropy and the environmental consequences for herbivores. Questions also arise about so-called transitive silencing, in which RdRp amplifies the RNAi signal throughout the plant, silencing genes in other plant tissues and organs. A study in *Arabidopsis* showed that RNAi can produce unexpected pleiotropic effects, such as reduced pollen viability, even when other aspects of plant growth seem to be normal [48]. In theory, vertical

gene flow of an RNAi-mediated pollen lethality phenotype to native plants could alter fitness, plant community composition and biodiversity. Some researchers have already begun to evaluate the potential for off-target effects. In their study in HD-RNAi nematode-resistant tobacco, Fairbairn *et al.* searched a genomic database for homologies between nematode and plant genes [29]. No homologies were found, so the authors suggested a low probability for off-target effects in this GE tobacco plant. However, this type of *in silico* approach for prediction of off-target effects will be limited by the availability of suitable genomic databases for plant species and the organisms interacting with them. Nevertheless, further research into off-target effects should be encouraged because the current lack of information creates uncertainties about this particular hazard.

### *Non-target effects*

As with Bt crops, it is possible that HD-RNAi pest-resistant crops could have harmful effects on non-target organisms exposed to living plants, plant parts or debris (Figure 1). For example, research has shown that insect pests consuming small RNA molecules could be killed (or stunted) by cleaving mRNA of the vacuolar ATPase housekeeping gene [25]. If there is sufficient homology between the housekeeping gene in the target pest and other non-target organisms (e.g. beneficial insects, other herbivores), unintended gene silencing could occur with negative consequences. Genomic databases and well-designed laboratory feeding studies might prove useful in determining the likelihood of such non-target effects. However, the lack of genomic databases for many non-target organisms could present a challenge.

### *Environmental persistence of small RNA molecules*

The potential for off-target or non-target effects of RNAi crops highlights the importance of characterizing the environmental fate of small RNA molecules synthesized in plants. Currently, very little is known about the persistence of extracellular small RNAs in the environment, although they are known to have natural functions in communication, symbiotic relationships and other processes [47,49]. Extracellular DNA has been found in aquatic and terrestrial environments, which persisted for months or even years (in soil) despite the presence of nuclease enzymes [49,50]. Absorption of DNA into complex organic molecules is believed to provide protection from nuclease degradation. Although some research has characterized environmental DNA, very few studies have addressed the persistence of RNA in different ecosystems. Bacterial biofilms are known to contain a complex mixture of molecules including single-stranded RNA. Extracellular RNA has persisted in blood stored on filter paper at 32 °C for 3 months [51]. In plants, extracellular RNA is known to move through the phloem and between cells, but its persistence in plant debris has not been studied. Small RNAs are not very abundant in RNAi and HD-RNAi crops and this might lead to the conclusion that the risk is low. However, small RNAs are active at very low concentrations, so this would need to be considered in an ERA. It is not known if certain small RNA sequences inherently increase or decrease environmental stability and persistence. Insect diets containing dsRNA variants showed that longer RNA molecules were more effective, possibly owing to persistence in the system [25].

### *Effects of mutations and polymorphisms*

Heritable genetic mutations (e.g. base changes, deletions, insertions) occur in all organisms including crop plants and their pests. In addition, polymorphisms (small variations in DNA sequences) also occur in individuals within a population [24,46]. Given this natural background of genetic mutations and polymorphisms, research is needed to characterize the unintended effects of such natural variations on RNAi in crop plants and pests. There are a number of scenarios in which mutations and polymorphisms could affect the efficacy and stability of small RNAs, including: (i) mutations in the GE crop that would alter the nucleotide sequence of the novel small RNA molecules and patterns of gene silencing, possibly creating off-target effects; (ii) mutations and polymorphisms in plant pest populations (e.g. viruses, insects), which might lead to resistance to gene silencing and decrease the protective properties of an HD-RNAi crop; and (iii) mutations occurring in non-target organisms (e.g. beneficial insects), which could increase their susceptibility to the pesticidal properties of the HD-RNAi crop. For example, the rapid evolution and high mutation rates of plant viruses might allow these pathogens to quickly become resistant to a HD-RNAi crop [18–21,52]. Viruses often exist naturally in mixed populations and HD-RNAi crops could create selective pressure for resistant strains. For insect-resistant HD-RNAi crops, it will be important to anticipate environmental concerns about genetic changes that lead to complementarity between small RNAs and mRNAs in insects exposed to the HD-RNAi crop. Research is urgently needed to evaluate these potential hazards with regard to their probability, time frame for occurrence, the effect of scale (local, regional and national patterns of crop production) and the potential severity of impact.

### *Tracking RNAi and HD-RNAi crops*

Crop identity preservation, monitoring and segregation are important to many stakeholders in the food chain, including biotechnology companies, seed producers, farmers, food manufacturers and exporters [53]. Regulatory agencies also need to be able to monitor and track GE crops if necessary. At present, GE crops such as herbicide-resistant soybeans and Bt maize are often detected using an easy and inexpensive ELISA procedure. Methods using ELISA strip tests and DNA-based PCR can detect Bt endotoxins at concentrations as low as 0.5%, although quantitative results are not reliable below 0.5% [54]. Without expression of a novel protein, ELISA strip tests cannot be used for RNAi and HD-RNAi crops and derived food products. Therefore, detection and monitoring will probably have to be performed in a laboratory using PCR and sequence-specific primers. This will not only increase the cost for many stakeholders, but will also eliminate rapid field testing. Although marker genes (e.g. antibiotic resistance, sugar isomerases) and their expressed proteins could serve as a basis for ELISA strip tests, there could be issues regarding specificity and discrimination among GE crops.

### Uncertainties

Epistemic uncertainty about what we do not know is inherent in any ERA and assessors are required to clearly indicate the extent of uncertainties in their analysis [55]. Naturally, there is greater uncertainty associated with novel technologies than with those that have an established track record. Protein-based GE crops have been commercially available for more than 14 years and risk assessment research into these crops goes back even farther. This research has not only improved our understanding of the mode of action of the transgenic proteins, especially the Bt endotoxin, but has also answered many questions for ERA [43,56]. Because RNA-mediated traits are still in their infancy, von Krauss *et al.* [57] evaluated uncertainty using interviews and expert opinion. They concluded that experts in the field had some uncertainty about how silencing mechanisms performed under varying conditions and over time, and there were discrepancies between experts about cause–effect relationships in gene silencing. When making decisions based on risk assessments, regulators and stakeholders need to balance the level of risk against the uncertainty associated with the risk assessments used. In many cases RNAi technology might present a low environmental risk overall, but if these low risks are perceived to have a high level of uncertainty, substantial testing and management controls might be required before commercialization is licensed.

## Concluding remarks

Recent advances have created high expectations for the future role of RNA-mediated traits in GE crops. Perhaps the most important applications will be in altering crop–pest interactions so that plants are protected from insects, nematodes or pathogens. Some researchers have extended this concept to the protection of humans and animals from disease. It has been suggested that plants could serve as biological factories for small RNAs that could become therapeutic treatments for viral pathogens in humans and animals [58,59]. However, substantial research is needed before the next generation of crop plants can be modified through RNAi to meet the needs of a growing human population. Because most RNAi research has been carried out in *Arabidopsis*, there are substantial gaps in our knowledge about the RNAi mechanisms at work in all of the economically important crops and host–pest interactions. For example, the parallel RNAi silencing pathways described in *Arabidopsis* (e.g. tasiRNA, natsiRNA) have not been clearly elucidated in most crop species [4].

In the future, the predictive ERA process will need to be flexible and adaptable for analysis of the next generation of crops engineered using RNAi and HD-RNAi. As a first step, regulatory agencies and risk analysts need to become familiar with the science of RNAi and its application to plant biotechnology. A concerted effort is needed to develop a pool of expertise to ask the right questions about potential hazards and exposures, to ensure that relevant data are collected and to characterize uncertainty in risk assessments. Regulators will have to evaluate the design and implementation of research protocols for laboratory experiments and confined experimental field trials. Scientific questions will need to be answered about off-target effects, non-target effects and the impact of genetic mutations and polymorphisms. Understanding the stability, persistence and half-life of small RNAs in various aquatic and terrestrial ecosystems will be essential for the characterization of exposure pathways. New diagnostic tools will probably be required for the identification and quantification of small RNAs for a range of purposes, including crop identity preservation, monitoring and segregation. Ideally, these tools should have a low detection limit and a high degree of specificity for each RNAi crop, while being relatively inexpensive, functional under field conditions and operable by individuals with diverse backgrounds and training. With all this in mind, it should be possible for stakeholders, regulators and citizens to develop policies and ERA frameworks for RNAi and HD-RNAi crops.

## Disclosure statement

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Review

# How to cope with insect resistance to Bt toxins?

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**Transgenic Bt crops producing insecticidal crystalline proteins from *Bacillus thuringiensis*, so-called Cry toxins, have proved useful in controlling insect pests. However, the future of Bt crops is threatened by the evolution of insect resistance. Understanding how Bt toxins work and how insects become resistant will provide the basis for taking measures to counter resistance. Here we review possible mechanisms of resistance and different strategies to cope with resistance, such as expression of several toxins with different modes of action in the same plant, modified Cry toxins active against resistant insects, and the potential use of Cyt toxins or a fragment of cadherin receptor. These approaches should provide the means to assure the successful use of Bt crops for an extended period of time.**

## Transgenic crops: an environmentally friendly alternative for insect control

Insect pests are one of the major problems in agriculture. Although chemical insecticides have been able to control these pests, their intensive use has created severe problems. Some chemical insecticides are recalcitrant and pollute the environment, and kill not only insect pests, but also beneficial insects and vertebrates, including people. Moreover, many insects have evolved resistance to chemicals, which has resulted in increased pesticide use. Since 1996, insect-protected transgenic crops, which are known as Bt crops, have been grown worldwide; they have proven to control insect pests efficiently and helped to reduce the use of chemical insecticides [1,2]. Bt crops produce insecticidal proteins from *Bacillus thuringiensis* (Bt) bacteria, also known as Cry toxins because they are produced as crystal inclusions. Cry toxins are classified based on primary sequence similarity into 54 types (Cry1–Cry54) and many sub-types (e.g. Cry1Aa and Cry1Ba). They are highly specific in that they are active only against a limited number of susceptible insects, including lepidopterans, coleopterans, dipterans, or against nematodes [3,4]. A major group of Cry-toxins is the three domain (3D)-Cry family, members of which share similarities in sequence and structure, but there are other groups of Cry proteins that are different from 3D-Cry (Figure 1). Despite the large number of Cry toxins, only about a dozen (Cry1Aa, Cry1Ab, Cry1Ac, Cry1C, Cry1D, Cry1E, Cry1F, Cry2Aa, Cry2Ab, Cry3A, Cry3B and Cry34/Cry35) are used commercially as sprays or in Bt crops.

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## Is insect resistance a major threat for the long-term use of Bt crops?

Evolution of resistance is a genetically based decrease in a population's susceptibility to a toxin [5]. Insects are able to evolve resistance to Cry toxins [5–22] (Table 1). Resistance is evaluated with laboratory bioassays estimating the resistance ratio, which is the $LC_{50}$ (concentration of toxin killing 50%) of a field-derived strain divided by the $LC_{50}$ of the susceptible strain [5,7]. Because of concerns that insects would evolve resistance to Bt crops, a resistance management tactic, the 'high dose plus refuge strategy', was mandated in several countries including the USA. [6]. The 'high dose plus refuge strategy' entails the use of Bt crops that express high concentrations of Cry toxins and the planting of refuges of non-Bt crops near Bt crops. Refuges are intended to maintain susceptible insect populations. Results from modeling studies suggest that the evolution of resistance can be delayed if: (i) rare homozygous resistant insects (RR) from Bt crops mate with abundant homozygous susceptible insects (SS) and (ii) if the heterozygous offspring from such mating (RS) are killed by the high Cry toxin concentration in Bt crops.

Global monitoring data of six lepidopteran pests has indicated that during the first decade of Bt crops, resistance evolved in some populations of *Helicoverpa zea* but not in any of the other five species analyzed [7]. Documentation of *H. zea* resistance to Cry1Ac includes evidence that 14 strains that were derived from Bt-cotton fields during 2003 to 2006 had resistance ratios >100, including two with resistance ratios of >1000 [7]. By contrast, the maximum resistance ratio was 1.2 for *H. zea* before Bt cotton was commercialized [7]. The data collected before and after commercialization of Bt cotton provided evidence that exposure to Cry1Ac led to resistance to Cry1Ac in some field populations. The patterns observed in the field are consistent with projections based on refuge strategy theory and suggest that refuges have helped to delay resistance [7]. It is possible to select several Bt-resistant populations in the laboratory; furthermore, two resistant populations have been found outside the laboratory – a population of *Plutella xylostella* in the field and a population of *Trichoplusia ni* in glass houses – both of which arose as a result of the application of Bt sprays [8–10] (Table 1). Despite this, physiological and/or ecological constraints have prevented resistant individuals from causing significant crop damage.

With the increasing use of Bt crops, particularly in less-regulated crop systems where refuges are not required,

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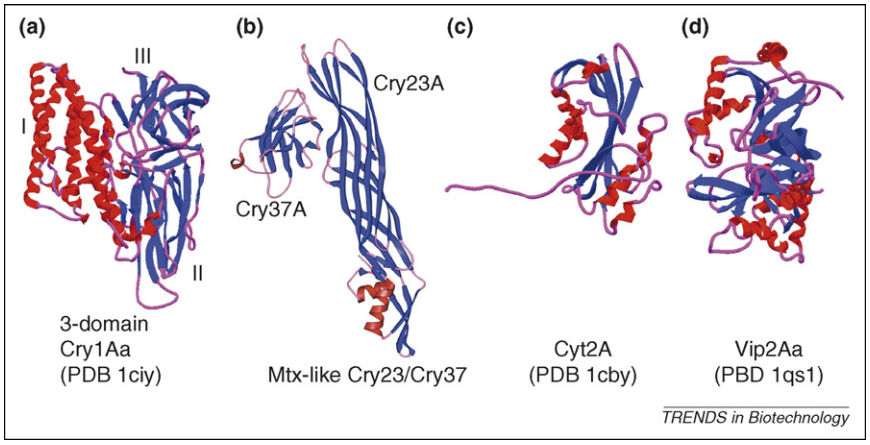


**Figure 1**. Three-dimensional structure of representative toxins produced by *Bacillus thuringiensis*. **(a)** The Cry1Aa protein is shown as an example of the three domain family Cry proteins. Domain I (in red) is the pore-forming domain and domains II and III (in blue) are important for toxin-receptor interactions. **(b)** The Cry23/Cry37 as an example of binary Mtx-like Cry toxins. The Cry23 toxin shares homology with the Mtx toxins of *Bacillus sphaericus*, which can kill mosquitoes, but the Bt-Mtx-Cry toxins are active against coleopteran insects. Image kindly supplied by T.J. Rydel, Monsanto Co. **(c)** The Cyt2Aa toxin as an example of Cyt toxins. **(d)** The Vip2Aa as an example of the Vip toxins. These proteins are secreted during vegetative growth. Vip1 and Vip2 constitute a binary toxin active against some coleopterans.

insect resistance is likely to become an increasing problem. Therefore, ways to counter insect resistance need to be developed and put in place soon. In the following sections we will review the principal mechanisms of resistance to Bt toxins and strategies proposed to deal with resistance in the field.

## Mechanisms for insect resistance to Bt toxins

To kill insect larvae, 3D-Cry toxins undergo a multi-step process that results in insect midgut cells bursting. Two different hypotheses for the mode of action of these toxins have been proposed, one relies on pore formation and the other on signal transduction (Figure 2). The first steps in both models are similar: the toxin crystals are ingested by the larvae and solubilized in the gut into protoxins. These are cleaved by midgut proteases to give rise to a 60-kDa 3D-Cry toxin that includes a helix α-1 at the N-terminal end (Figures 1 and 2) [4]. The activated toxin is able to bind to a cadherin receptor that is located in the microvilli of the midgut cells [23]. The pore-formation model proposes that interaction with cadherin facilitates further proteolytic cleavage [24], resulting in the oligomerization of the toxin. The toxin oligomer then binds to secondary receptors, which are proteins anchored to the membrane, by a glycosylphosphatidylinositol (GPI)-anchor, such as aminopeptidase N in *Manduca sexta* or alkaline phosphatase in *Heliothis virescens* [4,25,26]. In a final step, the toxin oligomer inserts into lipid raft membranes, where it forms pores and subsequently causes cells to burst, resulting in the death of the larva (Figure 2) [4,27,28]. By contrast, in

**Table 1. Description of some insect pests and nematodes resistant to Cry toxins**

| Scientific name | Common name | Main affected crops[a] | Resistance to Cry toxins | Mechanism of resistance | Refs |
|---|---|---|---|---|---|
| *Caenorhabditis elegans* | Nematode worm | - | Cry5B | Defects in glycolipid synthesis | [13,51] |
| *Culex quinquefasciatus* | Mosquito | - | Cry4A, Cry4B, Cry11Aa | Unknown | [11] |
| *Diatraea saccharalis* | Sugarcane borer | Corn, sorghum, sugar | Cry1Ab | Unknown recessive | [63] |
| *Ephestia kuehniella* | Mediterranean flour moth | Stored flours | Tolerance to Cry1A, Cry2A | Tolerance owing to overproduction of lipophorin | [20,62] |
| *Helicoverpa armigera* | American bollworm | Cotton, beans, corn, sorghum | Cry1Ac | Lack of cadherin receptor. Overproduction of esterases and hexamerin | [17–19,61] |
| *Helicoverpa zea* | Corn earworm | Corn, cotton, tobacco, tomato | Cry1Ac | Unknown | [64] |
| *Heliothis virescens* | Tobacco budworm | Cotton, corn, tomato | Cry1Ac, Cry2Aa | Lack of cadherin and alkaline-phosphatase receptors. Defects in proteases | [15,26,36,53,59] |
| *Pectinophora gossypiella* | Pink bollworm | Cotton | Cry1Ac, Cry1Ab | Lack of cadherin receptor | [16] |
| *Plodia interpunctella* | Indianmeal moth | Meals, flours, nuts | Bt subsp. *entomocidus* | Defects in midgut proteases | [52] |
| *Plutella xylostella* | Diamond-back moth | Brassicae, crucifereae | Cry1Ac, Cry1Ab | Unknown, recessive | [21,22] |
| *Spodoptera exigua* | Beet armyworm | Rice, sugar beet, cotton, tomato | Cry1C | Lack of aminopeptidase 1 | [54] |
| *Trichoplusia ni* | Cabbage looper | Brassicae, crucifereae | Cry1Ac | Unknown, recessive | [14] |

[a]'-' means there was no effect on crops.

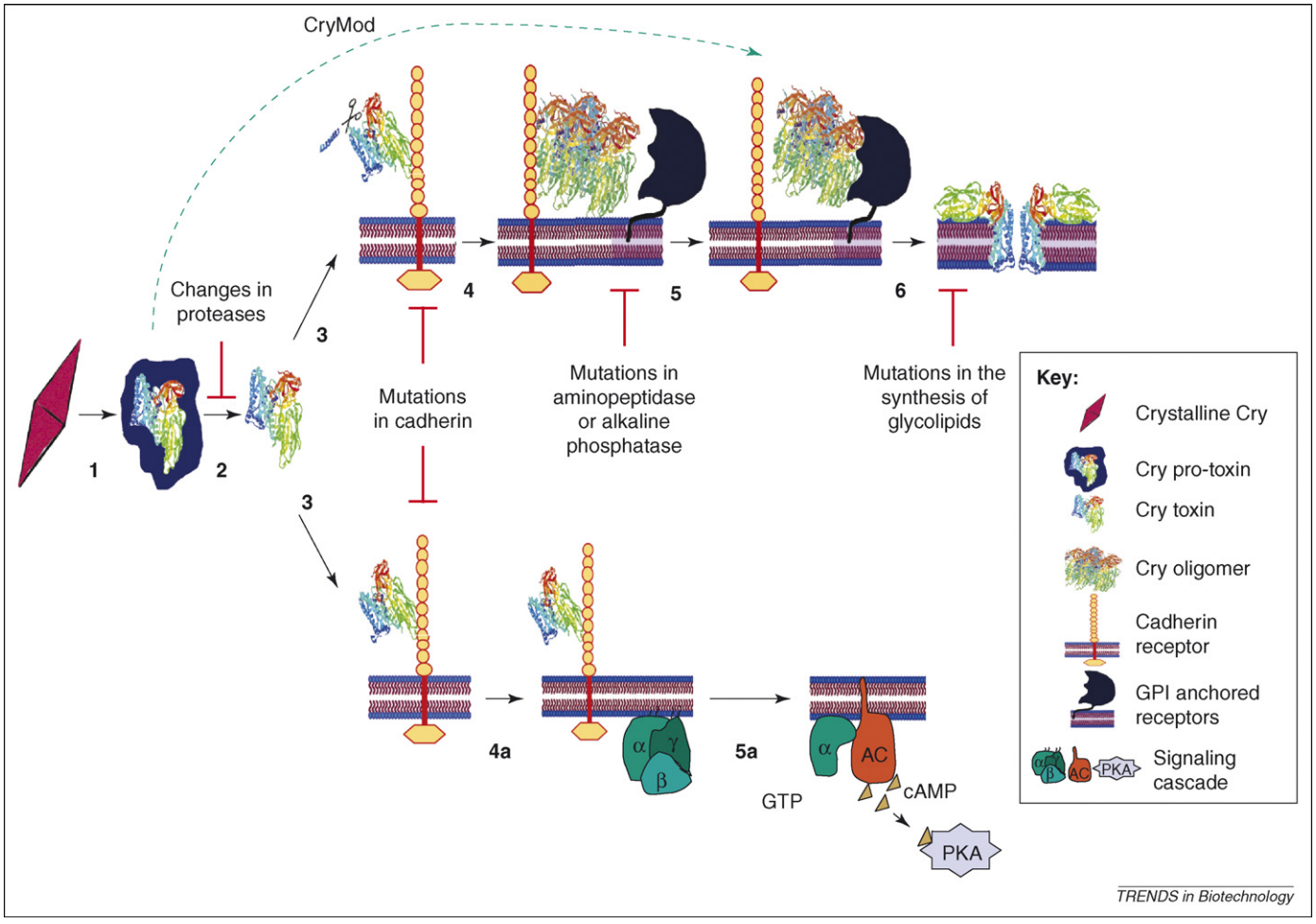


**Figure 2**. Models of the mode of action of Cry toxins and resulting mechanism for resistance. Two different mechanisms can be distinguished: the pore-formation model (top) and the signal transduction model (bottom), which both include similar initial steps for toxin solubilization in midgut lumen (1), activation by midgut proteases (2), and binding to primary receptor cadherin (3). In the pore-formation model (top), step 3 induces the cleavage of helix α-1 and triggers toxin oligomerization (4). The toxin oligomer then binds to a secondary receptor, such as aminopeptidase or alkaline phosphatase, which are anchored by a glycosylphosphatidylinositol anchor in the membrane (5). Finally, the toxin inserts itself into the membrane, thereby forming a pore that kills the insect cells (6). The signal transduction model (bottom) proposes that the interaction of the Cry toxin with a cadherin receptor triggers an intracellular cascade pathway that is mediated by activation of protein G (4a), which, in a subsequent step (5a), activates adenylyl cyclase. This signal then increases the levels of cyclic adenosine monophosphate, which activates protein kinase A and leads to cell death. See Refs [13,15–20,51–54] for the different mechanisms that have resulted in toxin resistance in several insects. The CryMod toxins, in which helix α-1 is deleted, avoid resistance by bypassing cadherin interaction [38].

the signal transduction model, the binding of Cry1A to cadherin is assumed to trigger a cascade pathway involving the stimulation of a G protein and adenylate cyclase to increase cAMP, resulting in the activation of protein kinase A, which in turn leads to oncotic cell death (Figure 2) [29]. The mode of action of 3D-Cry has been studied in different insect orders and nematodes (Box 1); the findings from most of these studies support the pore-formation model. The signal transduction model was proposed based on studies only performed in an insect cell line. In theory, disruption of any step of the toxin action pathway could cause insect resistance [8,9] and has indeed led to resistance to Cry toxins evolving in different insects and nematodes [11–22]. In Table 1, Box 2 and Figure 2, the proposed mechanisms of resistance are outlined. In general, the most frequently observed mechanism of Cry toxin-resistance involves defects in receptor binding, followed by resistance owing to defects in protease production, elevated immune response or enhanced esterase production [8,9,19,20]. Characterization of the resistance that occurred in some lepidopteran pests has revealed the so-called 'mode 1' type of Bt-resistance. This mode 1 resistance is characterized by a high level of resistance to Cry1A toxin, recessive inheritance, and also shows reduced binding of at least one Cry1A toxin as well as little cross-resistance to Cry1C [5]. In *H. virescens* [15], *Pectinophora gossypiella* [16] and *Helicoverpa armigera* [17,18], mode 1 resistance can be linked to mutations in the cadherin gene (Figure 3, Box 2). One exception is a *Plutella xylostella* population that evolved resistance to spray applications of HD1 Bt-strain that contained Cry1Aa, Cry1Ab, Cry1Ac and Cry2A toxins [10]. This population presented mode 1-type of resistance, but here the observed resistance was not linked to the cadherin gene [21], nor to any other gene encoding putative receptor molecules that have been described in other organisms, such as aminopeptidase-N, alkaline phosphatase or glycolipids [22] (Box 1). It is possible that this population benefited from mutations in other proteins that were able to function as the primary receptor for the toxin.

**Box 1. Mode of action of three domain (3D)-Cry toxins in different insect orders and in nematodes**

It has been proposed that members of the 3D-Cry family function by a similar mechanism of action given that they share sequence similarity and similar structures. The mode of action of these toxins has been studied in different insects orders but most progress has been achieved with Cry1A in lepidopteran insects [4], for which two models of their mode of action have been proposed: the pore formation and signal transduction models (see Figure 2). Recent data suggest that the pore-formation model, which is proposed to function in lepidopterans, also functions in mosquitoes and in coleopteran larvae because similar receptor molecules, such as cadherin in the case of mosquitoes [55], and glycosylphosphatidylinositol (GPI)-anchored receptors for coleopteran and dipteran larvae [56,57], have been implicated in Cry toxicity. In addition, dipteran- and coleopteran-specific Cry-toxins have been shown to form oligomers that are able to become inserted into membranes and to form pores [42,58]. It is possible that other proteins present in the insect also play an important role as receptors of different 3D-Cry toxins. In the nematode *Caenorhabditis elegans*, the binding of the Cry5B toxin to invertebrate-specific glycolipids that contain the core tetrasaccharide GalNacβ1-4GlcNacβ1-3Manβ1-4glc was shown to be a limiting step in the mode of action of this toxin [51]. The binding to glycolipids is also thought to be an important step in the action of Cry1A in lepidopteran insects [51] given that it constitutes the first interaction of Cry toxins with the membrane epithelium before the binding to the cadherin receptor. Alternatively, glycolipids could be crucial for the interaction of the toxin oligomer before membrane insertion given that the GPI-anchored receptors are located in membrane lipid rafts enriched in glycolipids [28].

## Bt plants with novel Cry toxins

Besides the 3D-Cry toxins, several Bt strains produce other Cry-toxins that have no sequence similarity with 3D-Cry proteins, including the Bin-like-Cry, Mtx-like-Cry and Vip toxins (Figure 1) [3,30]. It has been proposed that these toxins have a different mode of action to that of the 3D-Cry toxins and hence might be able to control insects that are resistant to 3D-Cry. For example, the Bin-like Cry34/Cry35 binary toxins, which are toxic to the coleopteran *Diabrotica virgifera*, have been successfully expressed in transgenic maize [31]. Furthermore, transgenic maize plants that produce the VIP3 toxin have been generated that can target several lepidopteran insects [32]. Although transgenic VIP3-plants are not yet commercially available, they could provide a way to counteract any potential resistance to the Cry1A toxins that are currently expressed by commercial Bt crops.

## Pyramiding or *cry* gene stacking

The concept of a 'pyramid' applies when two or more toxins with different modes of action, for example, they bind to different receptor molecules, are produced in the same plant. In this case, the possibility of generating resistant insects is diminished exponentially because multiple mutations would be required to lose susceptibility to both toxins [33]. In 2003, the first transgenic cotton plants expressing two 3D-Cry toxins, Cry1Ac and Cry2Ab, were tested: the dual-toxin was shown to be highly effective against lepidopteran pests [34]. These two Cry proteins are known to bind to different receptors and it was anticipated that the evolution of any insect resistance would be significantly delayed. In another example, the commercial release of transgenic maize with six stacked *cry* genes was recently announced [30]. This transgenic maize, named 'SmartStax', contains three *cry* genes (*cry*34Ab/*cry*35Ab and *cry*3Bb) encoding toxins that target coleopteran pests and three *cry* genes (*cry*1A.1.05, *cry*2Ab and *cry*1F) that encode toxins active against lepidopteran pests, in addition to two traits conferring herbicide tolerance [30]. Although, the 'pyramiding' toxins should be an efficient way of delaying resistance to Bt crops, it has been shown that this approach loses effectiveness when transgenic plants expressing only a single *cry* gene are grown in close proximity to Bt crops expressing two *cry* genes because insects resistant to both types of Bt plants were selected [35]. Therefore, it is crucial that pyramided Bt crops are planted in isolation from other Bt-plants. Furthermore, the characterization of two independent populations of *H. virescens*, which were resistant to Cry1Ac and cross-resistant to Cry2Aa, has revealed that resistance to different Cry toxins that bind to different receptors is possible [36,37]. This observation indicates that gene stacking might not be a 'one-fits-all' solution to counteract insect resistance to Bt crops.

**Box 2. Mechanisms of resistance to Cry toxins**

**Proteolytic activation of Cry toxins**

In *Plodia interpunctella* and *Heliothis virescens*, resistance to Cry1A was shown to be because of defects in midgut protease activities that affected the activation of Cry1A protoxins [52,59]. Such a mechanism of resistance would be compatible with both models for the mode of action of Cry toxins (i.e. pore formation and signal transduction, see Figure 2).

**Receptor binding**

In the case of the cotton pests *H. virescens* [15], *Pectinophora gossypiella* [16] and *Helicoverpa armigera* [17,18], Cry1A resistance has been linked to mutations in the primary receptor gene (i.e. in cadherin). This mechanism of resistance is also compatible with both models of the mode of action of Cry toxins. However, the observation that Cry1AMod toxins, which lack helix α-1 and hence bypass interaction with cadherin, are nevertheless toxic to the resistant larvae that lack cadherin, clearly favors the pore-forming model.

Other receptor molecules have also been implicated in resistance. In the *H. virescens* YHD2 resistant strain, a mutated cadherin allele was responsible for 40–80% of Cry1Ac resistance levels [15]. However, additional mutations linked to resistance in this strain affected the production of a glycosylphosphatidylinositol (GPI)-anchored alkaline phosphatase [26,53]. A *Spodoptera exigua* strain that is resistant to Cry1C was shown to lack the mRNA transcript encoding a GPI-anchored aminopeptidase N1 [54]. Silencing of aminopeptidase N of *Spodoptera litura* by RNAi resulted in tolerance of larvae to Cry1C [60]. The lack of GPI-receptors as the underlying reason for resistance is only compatible with the pore-forming model. Finally, in the case of the nematode *Caenorhabditis elegans*, resistance to Cry5B led to the identification of several genes (*bre*) that encoded glycosyltransferases that are involved in the synthesis of invertebrate-specific glycolipids [13].

**Esterase sequestration and elevated immune response**

An *H. armigera* Cry1Ac-resistant strain showed increased production of gut esterase, which has been implicated in chemical insecticide resistance owing to their ability to hydrolyze insecticidal esters and to sequester xenobiotics, bound and sequestered Cry1Ac toxin [19]. Feeding a sub-lethal concentration of Cry1Ac toxin to *Ephestia kuehniella* led to tolerance to Cry1Ac toxin that correlated with an elevated immune response associated with the production of pro-coagulants that recognize and form specific aggregates around pathogens or toxins, such as hexamerin for *H. armigera*, or lipophorin for *E. kuehniella* [20,61,62]. Both esterase sequestration and an elevated immune response are compatible with both possible mechanisms of the mode of action of Cry toxins.

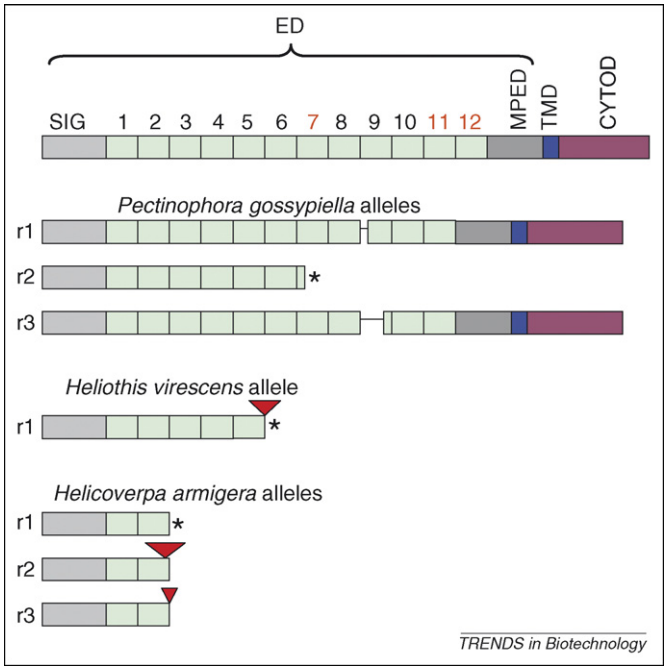


**Figure 3**. Gene structure of cadherin-alleles in different resistant lepidopteran species. The ectodomain (ED) is composed of a signal sequence for protein export (SIG): numbers correspond to cadherin repeats (CR) present in the ectodomain and membrane proximal ectodomain (MPED). The other domains are the transmembrane domain (TMD) and the cytosolic domain (CYTOD). The * indicates stop codons and the red triangle indicates retrotransposon insertion. In the case of *Helicoverpa armigera,* the smaller red triangle represents the long terminal repeat of a retrotransposon. Solid lines indicate deletions. Cadherin repeats 7, 11 and 12 are depicted in red because they contain the Cry1A binding regions that have been mapped on *Manduca sexta* Bt-$R_1$ cadherin receptor.

## Modified Cry toxins that bypass primary receptor interaction

As mentioned above, binding of Cry1A to cadherin facilitates the proteolytic removal of the helix α-1 of the toxin, thereby inducing toxin oligomerization and pore formation (Figure 2) [24]. In accordance with this observation, modified Cry1Ab and Cry1Ac toxins, which lacked helix α-1 (i.e. Cry1AbMod and Cry1AcMod) formed oligomers in the absence of cadherin [38]. Interestingly, these modified toxins killed *M. sexta* insects in which the cadherin protein was silenced by RNA interference (RNAi) and had resulted in tolerance to high concentrations of Cry1Ab. The modified toxins were also able to kill resistant *P. gossypiella* in which Cry1A-resistance had been found to be due to deletions in the cadherin gene (Figure 3) [38]. The Cry1AMod toxins bypassed the cadherin receptor and killed insect cells by inducing oligomerization and pore formation as depicted in the pore-forming model shown in Figure 2. However, it still remains to be determined whether Cry1AMod would be effective against insects for which resistance mechanism have not been linked to the cadherin gene. Furthermore, it will be interesting to test similarly modified versions of other Cry toxins that are active against other insect orders to determine whether this approach could have widespread applications. Finally, the effect of Cry1AMod needs to be evaluated under field conditions to verify whether Cry1AMod-crops could indeed withstand the attack of insect pests that have shown to be resistant to unmodified Cry toxins.

## *Bacillus thuringiensis* subsp. *israelensis* (Bti): a natural and efficient way to counter resistance to Cry toxins

*Bacillus thuringiensis* subsp. *israelensis* (Bti) strain is active against mosquitoes and produces four 3D-Cry toxins (Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa) and one Cyt1Aa protein. Each of these toxins shows low toxicity by itself, but using them in combination greatly increases their effectiveness in killing mosquito larvae and Cyt1Aa has been shown to be responsible for the synergistic activity of the other Cry toxins in Bti [4,39]. Despite the widespread application of Bti for more than 30 years, no resistant mosquitoes have been identified in the field to date. *Culex quinquefasciatus* populations resistant to Cry4 and/or Cry11Aa can be selected in the laboratory, but attempts to generate resistance to Cry11Aa in the presence of Cyt1Aa (3:1 ratio), or Cyt1Aa alone, have been unsuccessful [11,12]. Interestingly, resistance to Cry4 and Cry11Aa was overcome when these mosquito larvae were fed the Cry4 or Cry11Aa toxins in the presence of sublethal concentrations of Cyt1Aa [40]. In contrast to other Cry toxins, Cyt1Aa directly interacts with the mosquito membrane and does not bind to protein receptors [41]. In *Aedes aegypti*, Cyt1Aa is able to insert itself into the cell membrane where it functions as an additional receptor for the Cry11Aa toxin [39]. Cry11A binds to Cyt1Aa with high affinity ($K_d$ = 0.4 nM) with protein–protein interactions that involve specific regions of both toxins. The synergistic binding of both toxins correlates with an enhanced toxicity of Cry11Aa [39]. The interaction between Cry11A and Cyt1Aa facilitates oligomerization of Cry11Aa and its subsequent ability to induce membrane pores, which is a crucial step in the killing of the larvae [42]. Bti can therefore be considered as an insect-pathogenic bacterium that carries a toxin together with its functional receptor, thereby avoiding the occurrence of potential resistance owing to mutations in Cry-receptors [39]. To date, Cyt proteins have been found almost exclusively in Bt-strains active against mosquitoes. The only exception is Cyt2Ba, which is found in a coleopteran-specific Bt-strain [43]. However, it remains to be determined whether Cyt2Ba is toxic to Coleopterans and able to synergize the activity of other Cry toxins. In the case of lepidopteran insects, conflicting data have been reported. One study claimed that Cyt1Aa was toxic to *P. xylostella* and synergized with Cry1Ac in a resistant *P. xylostella*-strain [44], but other reports, also on *P. xylostella*, have shown that Cyt1A lacked any activity and, furthermore, did not synergize with Cry1A [45–47]. In any case, it would be interesting to identify Cyt proteins that are active against lepidopteran or coleopteran insects and that could act synergistically with other Cry toxins. As mentioned above, Cry11Aa binds Cyt1Aa through specific residues [39] and the genetic engineering of these regions is a potentially attractive and promising strategy to induce synergism between Cyt and Cry toxins in other insects and might become an important tool to overcome Cry-resistance by providing additional binding sites in the midgut membranes of resistant insects.

## Potential use of cadherin fragments to counteract insect resistance

Insect cadherins are modular proteins composed of three domains. The ectodomain contains the signaling peptide,

11 to 12 cadherin repeats (CR1 to CR12) and the membrane proximal ectodomain. The other domains are the transmembrane domain and the intracellular domain (Figure 3) [23]. Interestingly, a CR12-fragment expressed in *E. coli* that contained a Cry1A binding site enhanced Cry1Ab activity in different lepidopteran larvae [48]. It has been suggested that the CR12-fragment provided additional binding sites and increased the toxin concentration in the membrane given that it was localized in the microvilli membrane of the CR12-fed larvae [48]. Although the effect of the CR12-fragment has not yet been determined in any Bt-resistant population, it might potentially restore Cry1A toxicity in resistant insects, in which resistance has been linked to mutations in cadherin receptor.

## Conclusions and perspectives

In this Review, we discussed the different strategies that have been used to cope with insect resistance to Bt-toxins. Insect resistance is expected to occur in the near future based on the observation that resistant populations have been selected for in laboratory settings, and because lepidopteran insects have been identified that have already become resistant to Bt-toxins in the field [7,14,21]. The most frequently found mechanism of resistance involves mutations in toxin receptors, and resistance has indeed been linked to mutations in the cadherin gene in three cotton pests [15–18]. The different strategies that have been developed to date to cope with insect resistance rely on bypassing these receptor mutations, by introducing either Cry toxins that recognize different receptor molecules, or modified Cry toxins that can oligomerize and form pores in the absence of interactions with the cadherin receptor [30–34,38]. Another promising approach is the introduction of additional receptors, such as the development of Cyt toxins that might serve as a receptor for Cry toxins that are active against coleopteran and lepidopteran insects, or the administration of receptor fragments, such as the cadherin-derived CR12, which enhances Cry toxin activity in lepidopteran larvae. Finally, another potential approach to control resistant insects without the use of chemical insecticides is the targeting of essential insect genes by RNAi. Recent reports have shown that specific double-stranded RNA can be expressed in transgenic plants, which renders these plants resistant to insect damage [49,50]. It is likely that a combination of methods will be used in the future that will provide an effective means of protecting crops from insects without having to resort to the use of chemical insecticides.

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Review

# Chromosome engineering: power tools for plant genetics

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**The term ''chromosome engineering'' describes technologies in which chromosomes are manipulated to change their mode of genetic inheritance. This review examines recent innovations in chromosome engineering that promise to greatly increase the efficiency of plant breeding. Haploid *Arabidopsis thaliana* have been produced by altering the kinetochore protein CENH3, yielding instant homozygous lines. Haploid production will facilitate reverse breeding, a method that downregulates recombination to ensure progeny contain intact parental chromosomes. Another chromosome engineering success is the conversion of meiosis into mitosis, which produces diploid gametes that are clones of the parent plant. This is a key step in apomixis (asexual reproduction through seeds) and could help to preserve hybrid vigor in the future. New homologous recombination methods in plants will potentiate many chromosome engineering applications.**

## What is chromosome engineering?

Plant biotechnology uses genetic modification to create many useful traits. This innovation is layered on a constant background of conventional plant breeding, which will grow in importance as global climate change raises new challenges for agriculture [1]. Molecular markers generated by high-throughput sequencing will increase the efficiency of plant breeding [2]. However, the inherent slowness of combining favorable traits through genetic crosses and subsequent selection cannot be overcome by genomics alone. Chromosome engineering aims to create artificial chromosomes *de novo* or to change basic genetic processes by manipulating chromosome proteins. Tools created by chromosome engineering can greatly accelerate plant breeding. Artificial chromosome construction in plants has been summarized recently [3,4], thus this review focuses on methods that modify features of existing chromosomes to change their inheritance properties. Future applications in plant chromosome engineering that utilize homologous recombination technology are also proposed.

## Engineering centromeres to produce haploid plants

A fundamental difficulty in plant breeding is the need to produce functionally homozygous lines with consistent phenotypes (Figure 1). Molecular markers reduce the number of progeny that must be screened to recover useful trait combinations. However, several generations of selfing or backcrossing are required to create a new inbred. Once spontaneously arising haploid plants were discovered, geneticists realized that they offered a shortcut [5]. By producing haploids from a heterozygous parent, then converting them back into diploids (termed "doubled haploids"), breeders could rapidly make homozygous lines (Figure 1). Haploid production has revolutionized breeding in crops where it can be efficiently performed [5–7]. For example, hundreds of thousands of doubled haploid maize lines are produced each year. Haploids can accelerate genetic mapping and are beneficial for genomics because they remove heterozygosity. If haploid production is so useful, why has it not been universally adopted? To understand this question, we must explore barriers to haploid production by standard methods.

Regeneration of cultured haploid cells to yield adult plants is a widely practiced haploid production method [5,7]. Microspores (pollen precursors) are the most common starting material because of their higher number per flower, but ovules have also been cultured. These methods are efficient for a few species (e.g. canola or *Brassica rapa*) but have not worked in many important crops. Development of tissue culture protocols is largely empirical. In some species, phenotypic variation arising from tissue culture (termed "somaclonal variation") can be deleterious. Furthermore, regeneration is frequently too inefficient for production breeding and protocols are usually limited to a few genotypes.

A more biologically interesting haploid production method involves crossing a crop to a distant relative in an interspecific or intergeneric cross [5,7]. In a fraction of progeny, the genome from one parent is selectively eliminated after fertilization, yielding a haploid with chromosomes from the desired parent only. A classic example is the cross between cultivated barley (*Hordeum vulgare*) and *Hordeum bulbosum*, in which the *H. bulbosum* chromosomes are missegregated and lost during embryogenesis [8]. In many wide crosses, the seed is inviable and embryo rescue is needed to regenerate an adult plant. Maize haploid inducers, many derived from the classic "Stock6" line, are rare examples of an intraspecies cross that produces genome elimination [9]. Mapping of loci responsible for genome elimination in Stock6 has not yet identified genes that control the trait, although these efforts are narrowing down the genomic regions responsible for the phenotype [9,10]. As the mechanism underlying genome elimination in wide crosses is currently unknown, the phenomenon cannot be recreated in a new species.

A recent discovery in *Arabidopsis thaliana* suggests a completely new strategy for creating haploid plants [11]

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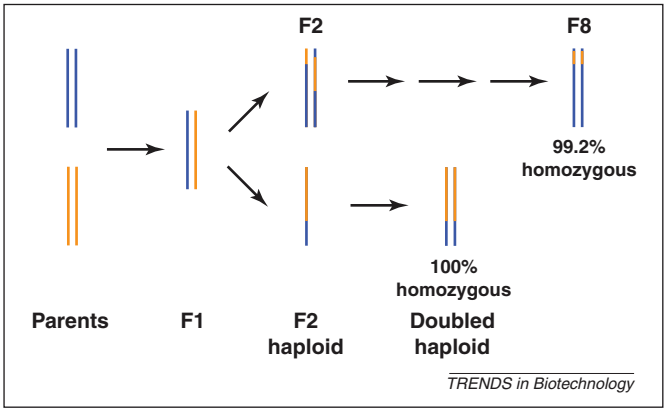


**Figure 1**. Haploid production accelerates conventional plant breeding. Traits from two different parents are combined in an F1 hybrid via crossing. Generations of inbreeding (e.g. F8 signifies the eighth generation since the original cross) are needed to produce functionally homozygous lines. Haploids have only one allele of every gene, thus if they can be converted back into diploids they can produce homozygous lines in a single step.

(Figure 2). Centromeres are loci that nucleate kinetochores, the protein complexes that bind to spindle microtubules and mediate chromosome segregation during cell division. In the novel method, centromeres are subtly disabled by mutating a kinetochore protein (such mutants must maintain chromosome segregation function to be viable). Crossing this centromere mutant to wild-type mixes two sets of chromosomes in the fertilized zygote. Chromosomes from the mutant parent (the "haploid inducer") have defective kinetochores and can be lost by missegregation during zygotic mitosis. Resulting adult plants are haploids with only chromosomes from their wild-type parent. This method mimics the genome elimination seen in wide crosses and potentially allows the process to be engineered into any plant.

In the published study, a haploid inducer was created by altering the essential kinetochore protein CENH3, a variant of histone H3 that replaces conventional H3 in centromeric nucleosomes [12]. Similar to conventional histone H3s, CENH3 has a C-terminal histone fold domain that complexes with other histones to form the nucleosome core and an N-terminal tail domain that protrudes from the nucleosome [13]. Unlike conventional histones, CENH3s evolve rapidly, particularly in their N-terminal tail. In *A. thaliana* haploid inducers, endogenous CENH3 was replaced by introducing transgenic proteins into a *cenh3* null mutant. The most efficient haploid inducer adds an N-terminal GFP tag to the protein and replaces the hypervariable tail of CENH3 with the tail of conventional H3 (termed "GFP-tailswap"). When *cenh3 GFP-tailswap* plants were crossed to wild-type, up to 50% of F1 progeny were haploid. All wide crosses described above produce a mixture of haploid progeny and diploid hybrids, in which chromosomes from both mutant and wild-type parents are kept (Figure 2). The frequency of genome elimination produced by GFP-tailswap in *A. thaliana* is higher than any previously reported wide cross. This suggests that centromere-mediated genome elimination might improve the efficiency of haploid production, even in crops such as maize and canola.

A key feature of the *A. thaliana* GFP-tailswap line is the ability to make either maternal or paternal haploids by crossing the mutant with female or male wild-type plants, respectively. Microspore culture produces haploids with paternal chromosomes and paternal cytoplasm. Crossing a CENH3-based haploid inducer (as the female) with a wild-type male shifts paternal chromosomes into the maternal cytoplasm. Cytoplasmic male sterility is useful for producing hybrid seed and facile cytoplasm exchange is likely to be one of the major applications of haploid inducers based on CENH3 alterations [14].

### *How can CENH3 engineering create a haploid inducer in crops?*

Endogenous *CENH3* must be inactivated or chromosomes from the inducer will not be outcompeted by those from the wild-type parent [11]. TILLING or insertional mutagenesis could create a *cenh3* mutation (such methods will be greatly aided by advances in high-throughput sequencing) [15]. Without a *cenh3* mutant, gene silencing methods,

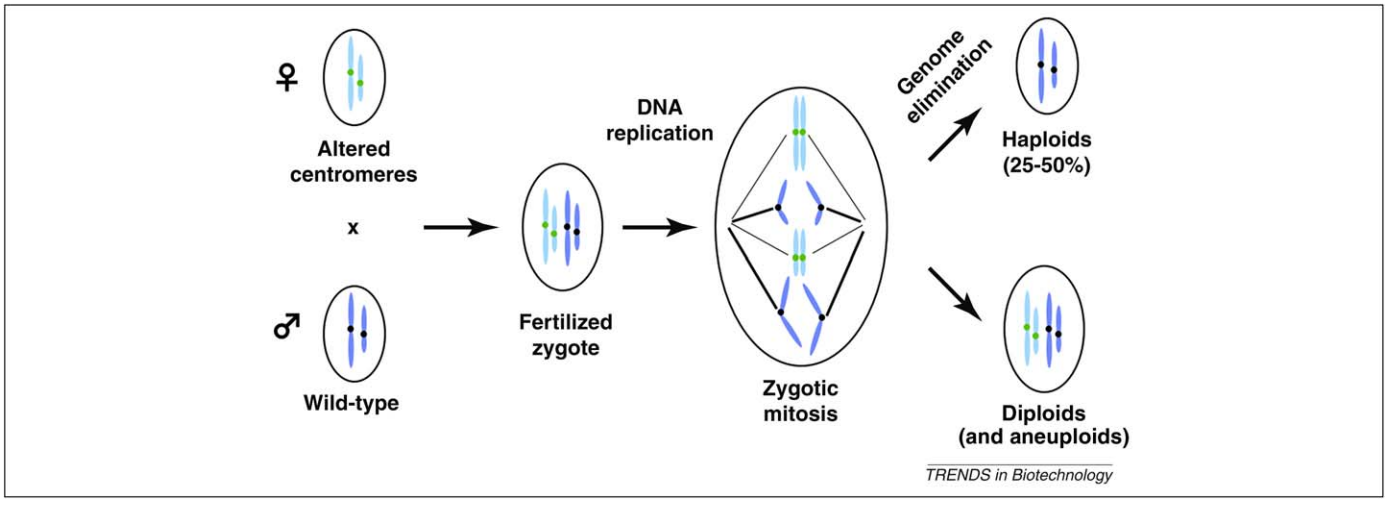


**Figure 2**. Altering centromeres to produce haploid plants. When a plant expressing an altered CENH3 protein (altered centromeres) is crossed to wild-type, its chromosomes (light blue) compete poorly during zygotic mitosis and are lost through missegregation in a process termed ''genome elimination''. A substantial fraction (25–50%) of adult plants can be haploids, with chromosomes from only their wild-type parent (dark blue). Note that the identity of the mutant and wild-type chromosomes must be maintained through DNA replication, presumably because pre-existing CENH3 remains associated with the kinetochore.

such as RNAi, should inactivate the endogene in any plant. The promoter for the RNAi transgene might need to be expressed in gametophytes, to ensure that endogenous CENH3 is absent from pollen or egg cell chromosomes (the commonly used 35S promoter is often poorly expressed in gametophytes [16]). Mutant CENH3 transgenes could be synthesized with altered codon usage to evade RNAi and should probably be expressed from the native *CENH3* promoter. CENH3 is a small protein, thus a single transgene can contain an RNAi transgene as well as a transgene expressing a mutant variant. Thus, a haploid inducer could conceivably be made in a single transformation. Haploids in *A. thaliana* were produced through seeds; as such, CENH3 engineering might avoid the need for tissue culture and, in some crops, potential somaclonal variation. Ideally, the method could offer haploid technology to breeders without access to highly standardized tissue culture facilities.

### *Can other centromere alterations create a haploid inducer?*

GFP-tailswap is not the only CENH3 variant that induces genome elimination. GFP-tagged full-length CENH3 also induces haploids, at a lower frequency, and many other alterations to CENH3 might cause missegregation in a cross. It has been suggested that alterations to the CENP-C protein could also cause genome elimination [17]. The potential for engineering other kinetochore proteins to produce haploids will depend on their behavior during DNA replication. After fertilization, both mutant and wild-type chromosomes are replicated during S phase, prior to the first zygotic mitosis. If a kinetochore protein is removed during DNA replication and reloaded onto both chromosome sets from a common pool, there will be no difference between chromosomes from the two parents and therefore no genome elimination. Pre-existing CENH3 at kinetochores is probably retained during DNA replication and partitioned equally between the two replicated sisters [18]. This explains why chromosomes from the mutant and wild-type retain their different behaviors, even if additional CENH3, presumably a mixture of mutant and wild-type protein, is loaded after S phase. CENP-C binds to centromere DNA directly, which might increase the chance that it remains associated with replicated chromosomes [19,20].

## Manipulating meiotic recombination frequency

A high meiotic recombination rate is useful for introgressing traits controlled by a small number of genes into another genetic background. Regions of the genome with suppressed recombination, often correlating with a high percentage of heterochromatin, pose particular difficulties. Forward genetic screens for mutants with elevated recombination are feasible in maize and *A. thaliana* using elegant genetic marker systems established to study local recombination. Kernel pigment phenotypes or fluorescent proteins expressed in pollen allow high-throughput scoring [21,22]. Reverse genetic approaches to increase recombination draw on meiosis research from yeast, mammals and plants. Meiotic recombination is initiated by double-stranded breaks catalyzed by the nuclease Spo11, which is broadly conserved in eukaryotes [23,24]. Processing of the double-stranded break can yield a crossover outcome (resulting in recombination) or a non-crossover repair event. Molecular understanding of this process is deepening, suggesting opportunities for engineering elevated recombination rates.

Two types of engineering could, in principle, elevate meiotic recombination. First, chromosome structure might be altered to allow easier access by recombination factors. Mutations that disrupt heterochromatin in the fission yeast *Schizosaccharomyces pombe* increase meiotic recombination near centromeres [25]. Although this is a promising approach, radical changes in chromatin structure might affect gene expression in unwanted ways. A note of caution is warranted based on comparing *A. thaliana rna-dependent rna polymerase2* (*rdr2*) mutants to maize *mop1* mutants in the orthologous enzyme. Both mutations reduce DNA methylation in the non-CG sequence context, yet *rdr2* has a very subtle phenotype, whereas *mop1* causes severe developmental defects [26–29]. This could result from the fact that the maize genome contains many more repeats, which could have evolved to play a larger role in gene regulation. In the future, it might be possible to produce local changes in chromatin structure, perhaps with engineered sequence-specific DNA binding proteins fused to enzymes that modify epigenetic marks (see discussion on engineered DNA binding proteins, below).

A second strategy for increasing meiotic recombination is to focus on recombination proteins themselves. In addition to Spo11, several other proteins that help to initiate recombination have been discovered through forward genetic screens (a majority of which were conducted in *A. thaliana*) and reverse genetic approaches using gene expression profiling to identify candidates [23,24]. Further explorations into meiotic crossover control are likely to yield practical insights. A recent study has discovered a *Caenorhabditis elegans* protein that regulates the crossover/non-crossover choice [30]. Furthermore, DNA helicases are key controllers of recombination rate in yeast [31–33] and manipulating such proteins in plants might increase meiotic recombination.

A related problem for plant breeders is introgressing traits from wild relatives that are so distantly related that chromosome pairing in meiosis I is difficult. Such homeologous pairing (between related chromosomes from different species) can be genetically controlled, as shown by the wheat Ph1 locus, which prevents recombination between homeologs [34]. The recent discovery that Ph1 downregulates cyclin-dependent kinases offers hope that the meiotic cell cycle machinery can be manipulated to allow homeologous recombination [35].

## Reverse breeding

A radically different method, termed “reverse breeding”, takes the opposite approach to the methods described above [36]. Reverse breeding suppresses meiotic recombination completely, resulting in the formation of gametes with various combinations of the intact chromosomes from either parent (Figure 3). Although meiotic recombination ensures accurate chromosome segregation during meiosis I, meiosis in the complete or near-complete absence of recombination will still yield rare, viable gametes. If these can be turned into adult plants by producing haploids (and

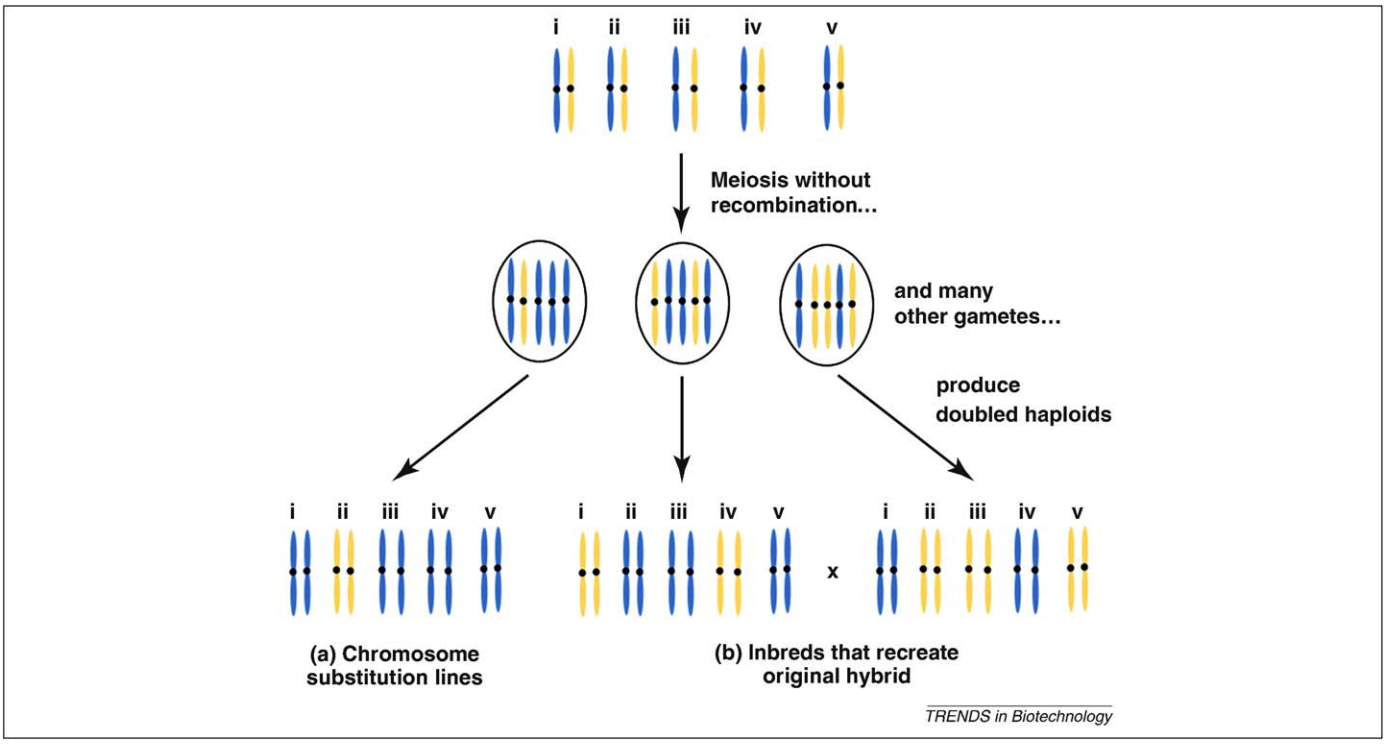


**Figure 3**. Reverse breeding can produce lines containing intact parental chromosomes, which allows heterozygous genotypes to be recreated. In reverse breeding, meiotic recombination is suppressed. Intact parental chromosomes (i–v) segregate into gametes, which can be converted into fertile plants by producing doubled haploids. **(a)** Single chromosomes can be transferred into an otherwise different genetic background. **(b)** Appropriate inbred lines can be crossed together to recreate the heterozygous genotype of the original parent.

subsequently doubled haploids), it is possible to create chromosome substitution lines in which a single chromosome from one inbred is transferred into the background of a different inbred parent (Figure 3). Such lines can be hugely valuable for trait mapping and introgression.

Heterosis (hybrid vigor) is a cornerstone of plant breeding [37]. Another application of reverse breeding is to start with an elite hybrid and create two inbred lines that will recreate the vigorous hybrid genotype when crossed (Figure 3). Reverse breeding to fix hybrid vigor is applicable to species with <12 chromosomes, because it is mathematically realistic to create complementary combinations of parental chromosomes for such plants [36]. Reducing meiotic recombination is straightforward given the large number of meiosis-specific proteins involved in this process. RNAi is an appealing method for downregulating recombination because it can be controlled by a conditional promoter. The combination of such methods with CENH3-based haploid inducers could make reverse breeding feasible for many crops in the near future.

CENH3-based haploid induction, reverse breeding and future methods to elevate recombination rate share an interesting feature: they are likely to involve transgenic plants in a breeding step but can produce completely non-transgenic progeny. It will be interesting to see whether such lines are classified as genetically modified organisms (GMOs), because they are indistinguishable from organisms that never had a transgenic parent. If doubled-haploid lines made using CENH3-based inducers or reverse breeding are not regulated as GMOs, it will be easier to market them where public resistance to transgenic foods is high, for example in Europe.

## Chromosome engineering for apomixis

Hybrid seeds have greatly increased agricultural productivity, but their genotype cannot be propagated through sexual reproduction. Asexual reproduction through seeds (apomixis) occurs in many plant species [38]. It is thought that apomixis alternates with sexual reproduction, allowing such plants to multiply favorable genotypes yet still create variation when necessary. Apomixis is often described as a potentially revolutionary technology for agriculture, because it could perpetuate vigorous hybrids indefinitely [38,39]; however, attempts to introgress the trait into crops have not succeeded. Furthermore, map-based cloning of genes that control apomixis has not yet identified individual loci responsible for the trait [40].

Although there are many ways for apomixis to occur in nature, a common route for scientists seeking to engineer it is to divide the process into three steps [39]. First, meiosis must be bypassed or altered so that the plant produces diploid gametes without recombination. The *dyad* mutant of *A. thaliana* was the first genetic lesion found to produce clonal diploid gametes, but the precise function of the DYAD/SWI1 protein in meiosis is not known [41,42]. Second, embryogenesis should begin without fertilization. Third, endosperm development must also be triggered without fertilization. Chromosome engineering has had notable recent success in achieving the first step [43].

A complex but efficient solution for creating clonal diploid gametes is to combine three mutations that affect meiotic chromosomes and meiotic cell cycle progression [43]. Removing the SPO11 nuclease prevents meiotic recombination. Chromosomes in *spo11* mutants segregate randomly in meiosis I, because they cannot pair with their homolog. In

meiosis I, sister chromatids normally segregate to the same side of the spindle, because their centromeres are held together by the meiosis specific cohesin protein REC8 [44,45]. When *spo11* and *rec8* mutations are combined, sister chromatids segregate to opposite sides of the spindle in meiosis I, effectively turning this division into mitosis. The final mutation, *osd1*, prevents the onset of meiosis II, leaving two diploid gametes with the same genotype as the parent plant (see Ref. [43] for diagrams illustrating the full process). *spo11 rec8 osd1* mutants are termed "MiMe", because they convert meiosis into mitosis. In MiMe plants, an astonishing 85% of female gametophytes and 100% of the pollen have the diploid genotype of the parent plant. The challenge of engineering apomixis now shifts to coaxing the diploid embryo sac to form a seed without fertilization. Prospects for solving this problem through developmental genetics have been reviewed elsewhere [46].

## Homologous recombination for chromosome engineering

Precise chromosome engineering using homologous recombination has tremendous potential for basic research and for biotechnology applications. A classic example is the engineering of balancer chromosomes in mouse [47]. Balancer chromosomes have an inversion that contains a recessive lethal mutation (Figure 4). They prevent recombination within the inverted interval and cannot be homozygous and are therefore very useful for maintaining mutations in a heterozygous state. In some crops, hybrid vigor can depend on a very small number of loci or even on single heterozygous genes [48]. If a counterselection against homozygotes can be achieved, engineering plant balancer chromosomes could be a way to preserve the advantages of heterosis without full apomixis. Balancer chromosomes inevitably result in partial sterility and this property will limit their application in crops where the seed is the product. However, balancers can be valuable research tools for plant genetics, even in these species.

Engineered translocations are another potential application in plants. The evolutionary history of karyotype rearrangement can be reconstructed, for example in the study that revealed how the base chromosome number of eight in the *Brassicaceae* was converted to the *A. thaliana* karyotype of five [49]. Normally, karyotype differences would prevent genetic exchange between two species; however, engineered translocations might restore sufficient synteny to allow productive recombination if the problem of homeologous pairing can be overcome. The converse approach is to create novel translocations to reproductively isolate a plant, a potentially useful application in crops where intercrossing with wild relatives is a concern.

Site-specific recombinases, such as Cre–Lox or FLP/FRT, can create precise chromosome insertions, deletions, translocations and inversions, and work well in plants [50]. A powerful use of site-specific recombination is to target transgenes to specific genomic locations [51]. This can be repeated through several rounds to allow "transgene stacking" or the insertion of multiple transgenes at the same locus [52]. Maize homologous recombination has been achieved recently by cutting the desired locus with a sequence-specific zinc-finger endonuclease and thereby enhancing recombination frequency [53,54]. This suggests that we can now integrate Lox or FRT sites in precise locations, bringing single-nucleotide accuracy to plant chromosome engineering and allowing the precise engineering of chromosome rearrangements (Figure 4). One limitation of homologous recombination is the cost of designing custom zinc fingers, but this is likely to decrease as the method is more widely adopted. The transcription-activator-like (TAL) class of plant pathogen effectors represents an entirely different class of modular sequence-specific DNA binding proteins [55,56]. TAL proteins contain short tandemly repeated domains, each of which recognizes a single base pair of DNA. Homologous recombination in *Drosophila melanogaster* has evolved from a method used by only a handful of laboratories to a routine technique [57]. A similar trajectory will allow chromosome engineers to create a new set of power tools for plant genetics.

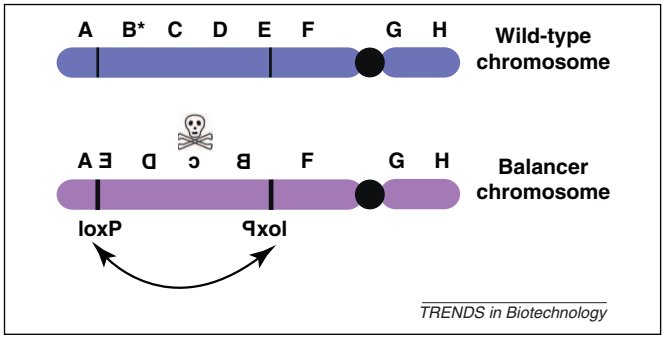


**Figure 4**. Homologous recombination allows precise engineering of balancer chromosomes. Balancer chromosomes contain an inversion relative to wild-type that prevents recombination in this interval (because recombination in this region creates dicentric and acentric chromosomes that are generally fatal to gametes). The letters A–H indicate loci on the chromosomes to show the position of the inversion. Balancer chromosomes can be constructed by integrating site-specific recombinase recognition sites (dark lines indicate loxP sites) in precise locations using homologous recombination. The balancer chromosome cannot be homozygous owing to a lethal mutation ('c') within the inversion. If B/B* comprise a pair of alleles that confers heterosis (hybrid vigor), balancers allow them to be maintained in the heterozygous state if the B*/B* homozygote can be selected against.

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