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, miRNAs differ from small interfering RNA (siRNA) in that they are usually produced from dsRNA. siRNAs 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.
- **RNA interference (RNAi)**: 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).
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.

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Table 1. GE crops in the USA using RNAia

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

*aDevelopment 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).

bVCPG, 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 (harm, 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...
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.
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 RdRpsi amplifies the RNAi signal throughout the plant, silencing genes in other organisms, often using a tiered approach [39]. Validation might be needed for tiered testing of crops with RNA-mediated traits. Persistence and fate of small RNAs in ecosystems (e.g. soil, water) are largely unknown. Extraction and identification of small RNAs for environmental monitoring can be very difficult.

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

<table>
<thead>
<tr>
<th>ERA information</th>
<th>Challenges and questions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bt endotoxin GE crop</td>
<td>Gene coding for Bt endotoxin protein</td>
</tr>
<tr>
<td>HD-RNAi GE crop</td>
<td>Gene coding for small RNA molecules (20–24 nucleotides)</td>
</tr>
<tr>
<td>Molecular characterization of active molecule</td>
<td>Limited genomic databases make comparative analysis for sequence homology in non-target species difficult</td>
</tr>
<tr>
<td>Mode of action</td>
<td>Multiple modes of action are known in Arabidopsis, but these are poorly understood in most crop species [9]</td>
</tr>
<tr>
<td>Toxicity testing</td>
<td>Lack of benchmarks or normalization for small RNA activity limits the ability to conduct comparative assessments [57]</td>
</tr>
<tr>
<td>Exposure assessment</td>
<td>Lack of normalized genomic libraries and RNA arrays for ecotoxicological model organisms [71]</td>
</tr>
<tr>
<td>Crop plant product sustainability</td>
<td>Persistence and fate of small RNAs in ecosystems (e.g. soil, water) are largely unknown</td>
</tr>
</tbody>
</table>

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 enzymes [49,50]. 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, natasiRNA) 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

The authors have no actual or potential conflict of interest with the material in this publication.

Acknowledgement

The views expressed in this paper are those of the authors and do not reflect the views of the US Environmental Protection Agency.

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