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RNAi Interference based protection of crops and their potential risks on environment

***Ghalia Nawal, Kausar Malik, Saira Ashfaq**

Department of Biotechnology Lahore, College For Women University
*Corresponding author: *ghalianawal@yahoo.com*

Abstract

RNAi interference is a posttranscriptional sequence-selective technique for the silencing of genes by suppressing gene expression in a sequence-specific manner.Molecular mechanism of RNAi. invloves member of the RNase III family, Dicer, and digested into 21 nucleotides siRNA duplexes. That forms RNA-induced silencing complex (RISC). The antisense strand of the siRNA then hybridizes to mRNA as a guide, and the RISC cleaves the mRNA near the center of the siRNA.Its high efficiency and ease of applications makes it a genome-wide analysis of gene function. In plants this phenomenon alters growth and development by stopping mRNA molecules from serving as the template for protein synthesis and suppressing phenotype expressions. An approach called host-delivered RNAi (HD-RNAi) is currently being developed for producing small RNAs that will silence essential genes in insects, nematodes and pathogens for crop protection by these manipulators.

Keywords: RNAi interference , RNA-induced silencing complex (RISC) and Host delivered RNAi.

Introduction

RNA interference (RNAi) is gene silencing technology that involves double-stranded RNA directed against a target gene or its promoter region. RNA silencing occurs in a wide variety of organisms, including protozoa, fungi, plants and animals and involves recognition of a target RNA and initiation of a sequence-specific RNA degradation pathway in the cytoplasm. This mechanism is conceived as a natural antiviral defense system in plants that is activated as a response to double-stranded RNA (dsRNA) formed during virus replication It is a posttranscriptional sequence-selective technique for the silencing of genes. Fragments of small RNAs (small interfering RNAs [siRNA] or microRNAs) bind to messenger RNAs (mRNAs) and promote cleavage by a complex of enzymes, thereby reducing the expression of specific genes. RNAi was originally known to occur in plants and fungi but was only first reported in animals (the nematode *Caenorhabditis elegans*) .

A cell produces double-stranded RNAs (dsRNAs) or microRNAs that target mRNAs from a specific gene, depending on nucleotide sequence, or dsRNAs are taken into a cell from the exterior environment (Huvenne and Smagghe, 2010). The dsRNA (generally fewer than 1000 nucleotides long is then cleaved into much smaller siRNAs (almost always 21–23 nucleotidet long), which are sometimes amplified intracellular .RNAi is not a way to knock out gene expression, only a way to suppress it, and sometimes only temporary.Using hairpin constructs, double-stranded RNA can be expressed in plants relatively easily, enabling this technology to be applied to a wide range of species to silence the expression of both specific endogenous genes and genes of invading pathogens to assist in crop protection. RNAi has also been used to engineer metabolic pathways to overproduce secondary products with health, yield or environmental benefits.

Guo and Kemphues first showed that injection of the sense or antisense RNA for a particular gene was able to suppress gene function in a sequence-specific manner.Later.Fire and Mello showed that it was in fact contaminating dsRNA in the sense and antisense preparations that was the real inducer of gene suppression in the study; this phenomenon was termed RNAi. Because dsRNA for introns did not show the RNAi effect, RNAi was thought to act in a post-transcriptional manner. RNAi-related phenomena had been demonstrated in plants before the discovery of RNAi by Guo and Kemphues. One of these phenomena is co-suppression, that is, gene silencing mediated by a sense transgene. In cosuppression, expression of the transgene itself is suppressed together with that of endogenous homologous genes. Co-suppression was subsequently shown to involve either transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS).

Another example of an RNAi-related phenomenon is coat protein mediated protection (CPMP). Virus resistance is conferred by a sense coat protein transgene. Initially, protection was thought to be induced by the coat protein, but later it was shown that untranslatable coat protein transgenes could also confer virus resistance. Because CPMP was found to act post-transcriptionally, it was thought that CPMP and PTGS shared similar mechanisms. Hamilton and Baulcombe made a striking discovery: they showed that the appearance of a small RNA molecule of about 25 nucleotides with homology to the target gene of PTGS was associated with the PTGS phenotype. A similar molecule was later found in an in vitro RNAi system for Drosophila and was named small interfering RNA (siRNA). These observations strongly suggested that PTGS and RNAi shared the same suppression mechanism and raised the possibility that dsRNA is generated during the PTGS process. It is thought that, in PTGS, ‘aberrant single-stranded (ss) RNA’ transcribed from a transgene triggers the generation of dsRNA by RNA-dependent RNA polymerase (RdRP), and consequently the RNAi pathway is activated .

Molecular Mechainism of RNAi:

In vitro RNAi systems for Drosophila have revealed the molecular mechanism of RNAi. First, long double stranded RNA is recognized by a member of the RNase III family, Dicer, and digested into 21 nucleotides siRNA duplexes. Each duplex is unwound and one of the two strands is incorporated, often preferentially, into the RNA-induced silencing complex (RISC). The antisense strand of the siRNA then hybridizes to mRNA as a guide, and the RISC cleaves the mRNA near the center of the siRNA. The siRNA duplex consists of a 19 nucleotide double stranded region with 2 nucleotide 30 overhangs Mismatches between siRNA and the target mRNA greatly reduce the efficiency of mRNA cleavage, particularly when these are located near the center of the siRNA . It is worthy to note that in plants a small number of mismatches can be tolerated.

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. 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. A. (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 30 untranslated region (30 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 . (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.

Tool for gene function analysis:

Although RNAi is not a knockout but a knockdown technology, its high efficiency and ease of application make it applicable to genome-wide analysis of gene function. In plants, RNAi is often achieved by a transgene that produces hairpin RNA (hpRNA) with a dsRNA region . Conventionally, antisense-mediated gene silencing has been widely used in the analysis of gene function in plants. Although antisense-mediated gene silencing is an RNAi-related phenomenon hpRNA-induced RNAi has been shown to be much more efficient. In an hpRNA-producing vector, the target gene is cloned as an inverted repeat spaced with an unrelated sequence and is driven by a strong promoter, such as the 35S CaMV promoter for dicots or the maize ubiquitin 1 promoter for monocots. When an intron is used as the spacer, which is essential for stability of the inverted repeat in Escherichia coli, the efficiency becomes very high: almost 100% of transgenic plants show gene silencing (Smith et al., 2000) . However, the mechanism by which the intron increases silencing efficiency remains unclear. RNAi can be used against a vast range of targets; 30 and 50 untranslated regions (UTRs) as short as 100 nucleotidest could be efficient targets of RNAi. For genome-wide analysis of gene function, a vector for high-throughput cloning of target genes as inverted repeats, which is based on an LR Clonase reaction, has been constructed(Wesley et al.,2001). Another high-throughput RNAi vector is based on ‘spreading of RNA targeting’ (also called transitive RNAi) from an inverted repeat of a heterologous 30 UTR (Brummell et al,2003). For analysis of genes essential to plant viability, a chemically regulated RNAi system has also been developed . Direct introduction of dsRNA or a plasmid producing hpRNA transiently by particle bombardment to induce RNAi in plants . This approach is useful for the analysis of gene function in plants in cases where transgenic approaches that require stable transformation are more difficult. Virus-induced gene silencing (VIGS) is another approach often used to analyse gene function in plants. RNA viruses generate dsRNA during their life cycle by the action of virus-encoded RdRP. If the virus genome contains a host plant gene, inoculation of the virus can trigger RNAi against the plant gene. Because this approach does not involve a transformation process, it might be suitable for the functional analysis of essential genes. Amplicon is a technology related to VIGS (Waterhouse and Helliwell,2003):. It uses a set of transgenes comprising virus genes that are necessary for virus replication and a target gene. Like VIGS, amplicon triggers RNAi but it can also overcome the problems of host-specificity of viruses.

Improvement of Crops:

Trait stability from one generation to the next is essential for the genetic improvement of crop plants. Phenotype suppression by PTGS may be inherited unstably. There are only a few reports describing the stability of hpRNA-induced RNAi. Phenotype suppression by hpRNA transgenes is inherited stably at least as far as the T5 generation in Arabidopsis. The rice mutant line LGC-1 (Low Glutelin Content-1) was the first commercially useful cultivar produced by RNAi (Kusaba et al.,2003). It is a low-protein rice and is useful for patients with kidney disease whose protein intake is restricted. This dominant mutation produces hpRNA from an inverted repeat for glutelin, the gene for the major storage protein glutelin, leading to lower glutelin content in the rice through RNAi. Interestingly, this mutant was isolated in the 1970s, and the mutant trait appears to have been stable for over 20 generations. These examples suggest that the suppression of gene expression by hpRNAinduced RNAi would be inherited stably. RNAi induced by hpRNA does not require some of the genes or components involved in PTGS, including RdRP (Beclin,2003). The reason why hpRNA-induced RNAi is inherited more stable than PTGS might be that hpRNA-induced RNAi does not require the generation of dsRNA mediated by RdRP for the suppression of gene expression. Downregulation can also be achieved through loss-offunction mutations. For rice, mutation-based reverse genetics and a gene targeting system are available (Miyao,2003).. RNAi has some advantages over these systems, however. One of these is its applicability to multigene families and polyploids (Lawrence, 2003), as it is not straightforward to

knockout a multigene family by the accumulation of mutations for each member of the family by conventional breeding, particularly if members of the family are tightly linked. Lowering of the glutelin content is achieved not by accumulation of loss-of-function of members of the glutelin multigene family (which comprises at least eight members, five of which are clustered in a particular chromosomal region), but from a single RNAi-inducing locus .

Another advantage of RNAi lies in the ability to regulate the degree of suppression. Agronomic traits are often quantitative, and a particular degree of suppression of target genes may be required. Control of the level of expression of dsRNA through the choice of promoters with various strengths is thought to be useful in regulating the degree of suppression. However, the use of a weak promoter appears to result in a reduction in the frequency of suppression, rather than the induction of weak suppression (Chuang and Meyerowitz,2003). An alternative approach is the use of sequences with various homologies to the target gene. In LGC-1, homology-dependent suppression by RNAi was observed (Kusaba et al.,2003). Such homology dependency could result from the effectiveness of each siRNA to cleave target mRNA(Sakamoto et al.,2003) The degree of suppression of a gene could be designed by using homologous genes isolated from closely or distantly related species that exhibit various homologies to the target gene. Such an approach could be applied to the improvement of various agronomic traits such as plant height and organoleptic properties. The control of tissue-specific or stimuli-responsive suppression is another possible application of RNAi, as the choice of suitable promoters could enable such regulation. However, gene silencing, not only by PTGS but also by the direct introduction of dsRNA, is known to spread systemically . This raises the possibility that when RNAi is induced in a particular tissue it might also suppress the target gene in other tissues where downregulation is not desired. A seed-specific promoter has been shown to be effective for suppressing constitutively expressed genes. Lgc1 acts as a Mendelian factor in F2 seeds on a single F1 plant, suggesting that there is no transmission of the silencing signal among developing seeds . Absence of plasmodesma between the seed and its surrounding tissues might affect the efficiency of spread of the silencing signal. Alternatively, the signal might be excluded from seeds, as it is excluded from the shoot apex. By such mechanisms, hpRNA-induced RNAi driven by a seed-specific promoter might confer seed-specific suppression; however, when other tissues, particularly where the PTGS signal travels easily, are specific targets of hpRNA-induced RNAi this specificity might be lost. In fact, systemic spread was observed in the chemically regulated RNAi system . This potential problem could be overcome by the use of a virus protein that suppresses the systemic spread of the PTGS signal or through knockout of a gene involved in the spread of the RNAi signal (Voinnet et al .,2000).

Protecting Crops:

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.

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 some pathogens.There is a growing interest in using RNAi for insect control, both as a traditionally applied insecticide and within Genetically Modifef plants. RNAi-based GM plants targeting insects have been developed in three independent research programs, although additional GM crops are in development.Baum and colleagues (2007) developed GM corn plants that resisted the western corn rootworm (*Diabrotica virgifera*; Coleoptera: Chrysomelidae). By reducing translation of vacuolar H+-ATPase subunit A (v-ATPase A) in the pest, the plant increased pest mortality and larval stunting and experienced less root damage as a result. Zha and colleagues (2011)transformed rice plants to suppress the expression of several genes in*Nilaparvata lugens* (Hemiptera: Delphacidae), a major pest. Although gene expression was suppressed, the insects were not killed by feeding on the GM rice plants. In a different approach to pest management, Mao and colleagues (2011) transformed cotton plants to produce double-stranded RNA (dsRNA) that reduced the expression of the P450 gene *CYP6AE14* in cotton bollworms (*Helicoverpa armigera*; Lepidoptera: Noctuidae). This P450 degrades gossypol, an antiherbivore phytochemical produced by cotton. GM cotton plants experienced less damage than the

conventional plants did, and the larvae that were fed the GM cotton had reduced growth but were not killed.

Risk Assessments:

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,2001) 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.. 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.. 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 RNAmediated traits 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.

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. Experiments with bacteria have demonstrated molecular crosstalk that decreased the expression of non-target genes (Whangbo. and Hunter, 2008). 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 (Xing and Zachgo, 2007). 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.,2007) searched a genomic database for homologies between nematode and plant genes . 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 But 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 . 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. If there is sufficient homology between the housekeeping gene in the target pest and other nontarget 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 (Vlassov et al.,2007). 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 . 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 8C for 3 months (Michaud et al.,2007). 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(Baum. et al., 2007).

Effects of mutations and polymorphisms:

Heritable genetic mutations like. base changes, deletions and 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. 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 (Soosaar et al.,2005). 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 crop:

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 (Auer, 2003). 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% . 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.

Conclusion

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. 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. 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.

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