Biosafety Briefing
November 2016

TWN Third World Network
www.twn.my

Risks of GM crops engineered to utilise RNA interference

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The development and release of genetically modified (GM) crops raises biosafety concerns for both humans and the environment. Producers of GM organisms (GMOs) are exploiting poorly characterised molecular mechanisms and techniques to engineer new traits into crops. One emerging biotechnology is based on the use of RNA interference (RNAi). While RNAi was discovered about 20 years ago, it is still not fully understood at the biochemical level.

Nevertheless, commercial products based on RNAi are being introduced. Such GMOs present a unique set of risks as compared with those generated with classical transgenic approaches. Already developed or currently in development are crops with insecticidal properties, resistance to viruses, altered product quality such as starch and acrylamide levels, reduced bruising (in potatoes), reduced allergenicity and lowered toxicant levels (e.g., gossypol in cotton). Some GM crops using RNAi are now being sold in the North American market, including the recent introduction of Innate® potatoes by J.R. Simplot Co.

Insecticidal crops are also close to release. MON87411 maize is glyphosate-tolerant and expresses a Bt toxin as well as a non-protein-coding RNA designed to downregulate a specific gene in the target corn rootworm pest, resulting in its lethality (see Box 1). Monsanto recently published on the purported human and ecological safety of RNAi in this maize.[1] [2]

This briefing covers the latest developments in the field of RNA interference that highlight the potential for RNAi GM crops to cause adverse effects, and the incomplete testing that has been performed thus far to address such concerns, using MON87411 as an example.

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Box 1: Examples of RNAi GM crops

To generate GM crops that utilise the RNAi pathway to target a given gene of interest, the process employed is like classical transgenic approaches, where DNA encoding dsRNA is introduced into the plant via, for example, *Agrobacterium*-mediated transformation, along with other DNA elements required for introduction and expression of the dsRNA construct.

MON87411 maize by Monsanto

MON87411 is a stacked maize event. It carries genes conferring both glyphosate tolerance and a Bt toxin (Cry3Bb1) targeting coleopteran pests. It also carries DNA encoding dsRNA targeting the *snf7* gene in corn rootworms, which encodes an essential vacuolar sorting protein. The theory is that the dsRNA is expressed into a ~1.2 kilobase RNA molecule, which is further modified into shorter, 240 base pair RNA inverted repeats that come together in a stem loop formation. When feeding on the corn, the rootworm is exposed to these dsRNA molecules, which are then recognised by the rootworm's RNAi machinery. Once the rootworm's RNAi machinery is activated, the dsRNA guides the machinery to the messenger RNA transcript of the *snf7* gene, inducing gene silencing through preventing protein translation, leading to eventual death.

Innate® potatoes by J.R. Simplot

Innate® potatoes produced by J.R. Simplot have been commercialised in the US and Canada. They carry up to four genes encoding traits for reduced starch (*pR1* and *pPhL*), reduced acrylamide (*asn1*) and reduced black spot bruising (*ppo5*). There is an urgent need for case-by-case assessment of such crops, where even the intended gene silencing may have adverse effects. For example, *asn1* (asparagine 1), introduced to reduce acrylamide levels, has been shown in studies to mediate plant pathogen defence.[38] Without thorough field studies to assess the risk of lowering the potato immune system, we are unable to ensure that such crops will not fail farmers once commercialised.

Introduction to non-coding RNA functions

The human genome project revealed that the vast majority (> 98%) of the human genome does not consist of protein-coding genes.[3] This phenomenon exposed the limitations of genetic determinism – the idea that individual genes determine how our body is constructed and what diseases we suffer from, as well as our patterns of behaviour and even intellectual ability. There were simply not enough genes to explain the complexity that exists in the human body, giving a clue to the existence of another level of gene function and regulation. To those within the genetic reductionist framework, the rest of the human genome was often thought of as having little function, being 'junk' DNA, filled with repetitive sequences, pseudogenes and old viral genomes, all evolutionary relics from the past.

A large proportion of this 'junk' DNA has since been found to be transcribed, but is not translated into detectable quantities of protein. This RNA has therefore been called non-coding RNAs (ncRNAs), comprising an estimated 50-70% of the entire transcribed genome.[4] An RNA revolution then followed,[5] with ncRNAs now being understood to accomplish a remarkable variety of functions in both plants and animals, from guiding genome rearrangement, to regulating gene expression, mediating cell-cycle control, cell identity decisions, protecting genomes from foreign nucleic acids, to functioning as enzymes and as a communication molecule with cross-organ and even cross-kingdom reach.[6] [7] [8] Double-stranded RNA (dsRNA) silencing is one such activity, largely functioning to mediate gene expression though repressing protein translation or direct modification of DNA and chromatin. Some of these changes are heritable (through epigenetic transmission), may result in persistent changes either within cells or entire

Box 2: RNA silencing

Double-stranded RNAs (dsRNAs) regulate gene function via several mechanisms; the most widely studied is RNA interference (RNAi). dsRNAs include miRNA (microRNA), siRNA (short-interfering RNA) and shRNA (short hairpin RNA). They are all key molecules that can induce RNAi, as well as chromatin-dependent gene silencing and post-transcriptional gene silencing. RNA silencing pathways are highly conserved across plants, vertebrates and invertebrates, mediating expression of endogenous protein-coding genes and non-coding DNA elements such as transposons, modifying DNA and chromatin structure and fighting viral pathogens. As such, RNA silencing is involved in almost every biological process in eukaryotes, and its dysregulation has been implicated in human disease.[39 40]

Mechanisms of dsRNA gene regulation

The underlying mechanism of RNAi gene regulation is understood to rely on the sequence complementarity of the small dsRNA molecules to its target mRNA of a given gene, resulting in gene silencing, through degradation of the target mRNA, or translational repression of the protein product, which is often the case when there is incomplete complementarity to the target sequence.

For RNAi, dsRNAs are typically formed when two complementary RNA strands are transcribed and come together to form a long dsRNA molecule, or from a long RNA molecule with stretches of complementary base sequences that come together to form a stem ending in a non-base-paired loop. These long dsRNA molecules are then processed into a shorter dsRNA (e.g., miRNA or shRNA), and one strand – the guide strand – directs a group of proteins to an mRNA, which interferes with translation.

Alternatively, the guide strand complex targets and chemically modifies DNA sequences in the nucleus by adding methyl groups to the DNA, and causes modification of histone proteins associated with the DNA.[36] The nuclear pathway is known to inhibit transcription and to seed the formation of heterochromatin, which are less actively transcribed regions of chromosomes. There is also emerging evidence of non-canonical RNAi pathways whereby dsRNAs can re-enter the nucleus to also regulate long non-coding RNAs and other miRNAs, modulating their biogenesis and function.[41]

dsRNA effects are not as specific as originally predicted, with as little as seven nucleotides of sequence identity (within a particular part of the mRNA, called the seeding region) being described as sufficient to induce activity (dsRNAs are typically between 20-30 nucleotides in length),[42] though even that requirement is being challenged.[43] Sequence data suggests some miRNAs can target many genes, while one gene can be targeted by many miRNAs, suggesting there is not always a simple one-to-one relationship between miRNAs and their targets, but a synergistic, interrelated relationship.[44] Indeed, small dsRNAs can target and regulate hundreds or even thousands of genes, with synthetic RNAs having an estimated 10% off-target effect,[45] despite being designed to target specific genes. It is therefore impossible to rule out off-target effects of dsRNAs unless thoroughly tested for.

tissues of organisms, and can be heritable through reproduction.

These functions are very different from messenger RNA (mRNA), which acts as the intermediate template for translating the mRNA of a gene into a protein. Mechanisms of RNA silencing are summarised in Box 2.

Such discoveries are contributing to a paradigm shift from reductionist thinking about autonomous organisms with precise boundaries, to embracing the more holistic concept of the 'hologenome' and 'holobiont', in which complex interrelations occur between living organisms of different species that live in intimate symbiosis, co-evolving

together.[8] This is of significance to the manipulation of living organisms through genetic engineering, where unpredictable and knock-on effects at the molecular level could have far-reaching ecological and human health implications.

Potential risks of dsRNA exposure

Persistence of dsRNAs in the environment

Often dismissed by regulators is the stability of dsRNAs, which, unlike messenger RNAs, are highly stable in the environment. Endogenous dsRNAs have been detected in nearly all extracellular bodily fluids including serum, plasma, breast milk and saliva. A 2016 *Nature* study of over 2,000 people detected over 1,000 dsRNAs in plasma.[9] Circulating dsRNAs are transported within extracellular vesicles such as exosomes, or in complexes with proteins or lipid-based carriers, which renders them relatively stable. Unlike messenger and synthetic RNAs which degrade rapidly, such circulating dsRNAs can be resistant to ribonuclease digestion, multiple freeze-thaw cycles, high and low acid conditions, boiling and extended storage.[10-13] They are now being explored as potential blood biomarkers for disease including cancers,[14] and for their active role in disease states.[15]

Plant dsRNAs are also chemically modified differently from mammalian dsRNAs. They are naturally methylated (2'-O-methyl groups) on the ribose of the final nucleotide, rendering them stable in serum, without affecting their RNAi activities in mammalian cells. A recent study chemically modified mammalian dsRNAs to include these methylation patterns; when fed to mice, these were successfully taken up in the intestine and subsequently reduced intestinal tumour burden.[16] It cannot therefore be assumed that the survivability and function of plant dsRNAs are the same as their unstable synthetic or mammalian counterparts.

Heritability of dsRNAs has also been shown in aphids exposed to dsRNA from plants, showing transmission through generations, which raises a serious concern over the safety of such products.[17]

The persistence and transmissibility of dsRNAs suggest potential for existence of exposure routes to non-target organisms. Assuming dsRNAs to be unstable and short-lasting is ignoring the most recent data on this topic.

Potential routes of exposure

Of relevance to RNAi GM crops is potential functional activity of transgenic dsRNA in non-target organisms. There are numerous examples of cross-kingdom communication, including between hosts and eukaryotic pathogens, pests, parasites or symbiotic microorganisms.[6 7 8]

Potential for cross-kingdom regulation between plants and higher organisms is evidenced by detection of exogenous RNAs in human circulation. Plant-derived (including from rice, corn, barley, tomato, soybean, wheat, cabbage, grapes and carrot), fungal as well as bacteria-derived dsRNAs have been detected in humans.[18] The functionality of such exogenous RNAs in mediating gene expression has been controversial, but there is an emerging consensus that such RNAs have functional capacity.[6 7 8] The pioneering work of Zhang et al.[19] not only showed the detection of rice miRNAs in circulation in animal blood, but also showed that they mediate gene expression following consumption. Looking at six mammalian species, including humans, they detected selective uptake of 30 miRNAs from rice. One in particular, mi168a, went on to mediate expression of a liver gene (LDLRAP1) related to cholesterol, leading the authors to speculate whether dsRNAs are indeed a nutrient.

These findings were rapidly followed by the publication of industry-sponsored studies that failed to replicate Zhang's work. Monsanto, in its petition to the US Department of Agriculture (USDA) for deregulation of MON87411, cited two such studies including

one by Witwer,[20] which failed to detect plant miRNA. This study used two animals only, compared with that of Zhang's team which included six animals of five additional mammalian species including humans (10 women, 11 men and a pooled serum from 10 extra individuals). The Witwer study only looked at seven plant miRNAs, whereas Zhang's team looked at global RNA levels using high-throughput sequencing analysis. Monsanto cited a second study that again failed to detect plant RNA in mammals after assessing only a few target miRNAs.[21] Further, in a communication published in *Nature Biotechnology*, Monsanto claims to have conducted its own experiments and failed to detect exogenous rice miRNA in mice.[22]

However, failure to detect anything is not proof that the exogenous RNA is not present in the mouse, especially when methodologies are narrow. As pointed out by Zhang in response to the Monsanto publication,[23] their positive control, which was rice miRNAs, was below the expected levels and hence minimised the chance of detecting anything in the mice.

Evidence has since accumulated on the presence and functionality of plant-derived dsRNAs, but this has been dismissed or even ignored in the latest publications by Monsanto claiming safety of MON87411.[1] [2] Zhang et al. have since published work showing that a dsRNA from honeysuckle, a traditional Chinese medicine, was taken up by mice and was able to target influenza strains including H1N1 and reduce severity of infection.[24] A 2014 study showed the presence of brassica vegetable miRNAs in serum, faeces, stomach and intestines, liver and kidneys of mice.[25] In a first-of-a-kind study published this year, broccoli miRNA not only was detected in mice, but was able to mediate gene expression to the extent that it reduced disease burden, in this case breast cancer, consistent with broccoli's reported anti-tumorigenic properties.[26] dsRNAs in bovine breast milk were also sufficient to mediate expression in human and murine cells *in vitro*, at nutritionally relevant doses.[27]

With regard to ecological risk assessment, dsRNAs can readily pass through the skin of worms, can be orally absorbed by honeybees and, in some cases, can even be amplified within the exposed organism, leading to more and different dsRNAs (secondary dsRNAs), with unpredictable targets. Secondary RNAs have been documented in plants, worms and fungi. It is possible that secondary dsRNAs are produced not only in the GM crop but also in the animal exposed to it. Of biosafety concern is that neither their identity nor their consequences can be predicted. As Heinemann et al.[28] stated in relation to the characterisation of dsRNA crop risks and the need to improve the risk assessment process: 'These secondary dsRNAs may have gene regulatory activities and thus act like siRNA. This means that dsRNAs created by the genetic engineering of plants may cause the production of additional unintended or unanticipated dsRNA molecules in both the genetically engineered plant and in any organism that is exposed to it…'

In mammals, other potential routes of exposure include the lungs via inhalation, or direct contact with skin or mucosa.[29]

Unsubstantiated safety claims of utilising RNAi for genetic engineering

Considering the above evidence of dsRNA function and downstream effects that can cross species, the purported claims of RNAi GM crop safety are outdated. The US Environmental Protection Agency (EPA) stated in 2014: 'Uncertainties in the potential modes of action in non-target species, potential for chronic and sublethal effects, and potential unintended consequences in the various life stages of non-target organisms are sufficient justification to question whether the current Agency framework for ecological effects testing is applicable to dsRNA PIPs [plant incorporated protectants] or exogenously applied non-PIP end-use products.'[30]

The new toxicology and ecotoxicology tests published earlier this year on MON87411 by Monsanto[1] [2] still do not address the latest evidence as raised by the EPA back in 2014. The limitations of these studies are summarised below.

Failure to address survival and activity of DvSnf7 from MON87411 in non-target organisms

The accumulating evidence that dietary dsRNAs not only survive digestion but may elicit gene regulation activity in mammals and other non-target organisms is acknowledged by the EPA, which states that considerable uncertainty remains with regard to survival of dsRNA in mammals. As such, it 'recommended experimental testing of the mammalian blood and exposed tissue be done to ensure that the siRNAs processed from the PIP dsRNAs are not present'.

However, no experiments were performed by Monsanto to look for survival or activity of DvSnf7, the dsRNA molecule introduced into its MON87411 maize, in non-target organisms. Considering data showing that consumed dsRNA can modulate gene activity following consumption, it would be logical to first look for DvSnf7 in organs and bodily fluids. Global gene expression studies would also be the most obvious assessment of the potential functional activity of DvSnf7 on mammalian genes using untargeted profiling approaches such as high-throughput sequencing analysis.[28]

Instead, the Monsanto study by Petrick et al.[1] consistently claims a lack of data replicating the initial work by Zhang et al.[19] detecting functional rice dsRNA in mammals and many other studies that have followed this. The 28-day mouse experiment was limited to gross examinations including 'weekly detailed observations, weekly body weights (unfasted) and final body weights (fasted for relative organ weight evaluations), weekly food consumption, serum chemistry, hematology, gross examination at necropsy, organ weights, and microscopic examination of tissues'. None of these parameters are sufficient to assess sub-lethal effects of dsRNAs. Short-term studies are also of far too limited duration to catch all relevant effects.

The study was also performed with DvSnf7 produced *in vitro*. Testing the synthetic version of DvSnf7 has limited relevance when one considers that it would lack the chemical modifications (e.g., 2'-O-methyl groups) which would occur in the plant and which are known to stabilise and promote dsRNA uptake by mammals. It would also underestimate the potential for selective packaging into micro-vesicles or other vehicles that could protect miRNA from degradation. Monsanto has published a study claiming the equivalence of *in vitro*- and plant-produced DvSnf7, though it did not test the methylation patterns, selective packaging and other factors affecting miRNA stability. The study was also narrow, assessing miRNA in corn rootworm larvae, not mammals or any other species, therefore ignoring all the issues surrounding survival in the mammalian digestive tract, for instance.[31]

It is not known what potential pleiotropic effects DvSnf7 could have on the maize genome (or indeed the other transgenic traits), which could potentially generate altered gene expression levels, novel nucleotides and genome scrambling, risks that also come with standard GM techniques and that could change the safety and nutritional status of the GMO.

As with the mammalian toxicity tests, the ecological assessments were largely performed using *in vitro*-produced DvSnf7 RNA. No experiments were performed to assess survival or activity of DvSnf7 in non-target organisms.

Other potential routes of exposure, including the lungs via inhalation, or direct contact with skin or mucosa, were also not assessed.

Failure to address the potential targets of the dsRNA from MON87411

The mammalian toxicity study used bioinformatics to analyse the potential complementarity of endogenous maize dsRNAs to human, rat and mouse mRNA. This was performed to claim that RNAs are 'generally recognised as safe' (GRAS). However, the properties of nucleic acids are determined by their sequence, such that sequence-specific effects cannot allow for GRAS. Moreover, unanticipated off-target adverse effects can be difficult to detect and are not possible to reliably predict using bioinformatics techniques alone. It is relevant to analyse potential matches of the dsRNA molecule introduced into the maize (DvSnf7) to mammalian messenger RNA transcripts. For this, Monsanto's analysis only searched for human, mouse or rat mRNAs that had a 100% sequence identity to DvSnf7, with the conclusion that no matches were detected. Similarly, only 100% sequence identity searches were performed in the ecotoxicology test on non-target organisms.

As outlined in Box 2, complete sequence identity is not necessary to induce RNAi activity, and sequence identity alone is not sufficient to predict activity of dsRNAs. This is even stated in the discussion of the paper, but used as an excuse for the lack of assessment of potential dsRNA targets. Further, they only searched for matches to the long pre-processed version of DvSnf7, instead of the shorter, processed version that would be present in the GM crop if the DvSnf7 transgene indeed activates RNAi. Establishing the shorter-form processed dsRNA of DvSnf7 that is generated would allow for establishing the positions of the seed sequences, making possible predictive analysis of potential targets that occur with less than 100% sequence identity.

They further state that due to the significant biological barriers that prevent the survival of dsRNA in mammals, such investigations are of limited value. However, as highlighted above, it is now widely accepted that plant RNAs do survive digestion. Predictive analysis of gene regulation is not sufficient to prove a lack of DvSnf7 activity following consumption. Such bioinformatics predictions need to be corroborated by experimental evidence.

Sequence-independent effects are also a source of toxicity for oligonucleotide therapeutics,[32 33] which should be tested with the plant-derived DvSnf7, as opposed to *in vitro*-produced DvSnf7, whose survival and stability in mammalian organisms remains to be determined. Sequence-independent activation of cellular sensors of foreign RNA and their downstream effects have been documented, including immune activation and cell death.[34 35] The latter effect depends mostly on the length – with toxicity linked to increasing nucleotide length, e.g., molecules over 30 nucleotides long – as well as structure, chemical modification and cellular localisation of the reagent, rather than on its sequence features.

There are many questions remaining on the genomics and physiology of higher organisms and wider food webs that may be highly exposed to dsRNA. These shortcomings in understanding currently preclude our ability to assess the reach of RNAi activity and to determine whether toxicity assays are sufficient to predict risks. With evidence of plant hosts communicating with various pathogens and symbionts including viruses, bacteria and fungi, the irrelevance of lab-based tests is also exposed. As detailed by Heinemann et al.,[28] risk assessment currently falls short of predicting all potential risks of dsRNA to non-target organisms.

Conclusion

The evolving field of RNA research raises concerns about GM crops utilising the RNAi pathway, concerns that remain to be fully addressed by current risk assessment studies. This has prompted scientists to raise concerns regarding their safety.[36 37] Under the Precautionary Principle, when there is reasonable suspicion of harm, lack of scientific

certainty or consensus must not be used to postpone preventative action. As argued above, there is indeed reasonable suspicion of harm even if consensus is lacking; RNAi GM crops such as MON87411 should thus not be approved.

Acknowledgement

I am grateful to Prof. Jack Heinemann from the Centre for Integrated Research in Biosafety, University of Canterbury, New Zealand, and Dr. Sarah Agapito-Tenfen from GenØk – Centre for Biosafety, Norway, for reviewing this paper.

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